MONIQUE DOSSENA ACAUAN

EFEITO DA TERAPIA LASER DE BAIXA POTÊNCIA EM GLÂNDULAS PARÓTIDAS DE CAMUNDONGOS SUBMETIDOS À RADIOTERAPIA

Dissertação apresentada à Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul como parte dos requisitos para obtenção do título de Mestre em Odontologia, área de concentração em Estomatologia Clínica.

Orientadora: Profa. Dra. Fernanda Gonçalves Salum

Co-orientadora: Profa. Dra. Ana Paula Neutzling Gomes

Porto Alegre

2015

EPÍGRAFE

“A verdadeira viagem de descoberta não consiste em buscar outros lugares, mas em olhar com outros olhos.”

Marcel Proust

AGRADECIMENTOS

AGRADECIMENTOS

Acima de tudo, agradeço a ***Deus***, quem me deu o dom da vida e quem me faz sonhar e me dá condições para tornar meus sonhos em realidade. A Ele que coloca pessoas tão importantes na minha vida, cada uma com um propósito diferente, tornando-a ainda mais especial. Sou grata a Ele, pois sei que sem Ele nada seria possível, e que com Ele nada se torna impossível.

Agradeço aos ***meus pais***, pelos valores que desde pequena me passaram, por me ensinarem a importância do aprendizado e a ter comprometimento com os estudos, por serem exemplos de dedicação e por darem o melhor de si e nunca medirem esforços para me apoiar e ajudar, não apenas nestes últimos dois anos, mas durante toda minha vida.

Agradeço a ***minha avó e minha madrinha*** por terem me acolhido e me fazerem sentir tão amada e cuidada como fui, no momento que mais precisei.

A ***meus irmãos, tias e a todos os meus familiares***, simplesmente por serem importantes para mim.

Ao ***Thiago***, pelo carinho, pelas palavras de incentivo, por me tranquilizar, por me distrair e tornar tudo mais leve quando estou ao seu lado.

Aos ***meus colegas da Estomatologia Clínica***, por podermos dividir momentos de alegria e tensão juntos, pelas trocas de escala e prestatividade. Apenas por ter conhecido e convivido com pessoas como vocês, todo o curso já teria valido a pena.

Agradeço àqueles que me cederam seu tempo e me auxiliaram na realização da fase experimental da minha pesquisa.

Ao ***Serviço de Estomatologia***. Às ***professoras Maria Antônia Z. de Figueiredo e Karen Cherubini***, por todo ensinamento e conhecimento transmitidos desde a época da faculdade. Tenham certeza que o exemplo que foram como professoras contribuiu de forma significativa para que eu optasse pelo mestrado em Estomatologia. Também à ***Márcia*** por todo auxilio que nos dá durante os dias de ambulatório.

Às ***professoras Fabiana Vier Pelisser e Maria Martha Campos***, por aceitarem o convite de avaliar meu trabalho durante a qualificação, e pelas ótimas contribuições que deram para o meu projeto.

A ***todos do SERP***, por me permitirem utilizar as dependências do serviço, em especial ao ***Dr. Aroldo Braga Filho***, e também a ***Daniela (física) e Miriam (técnica)***, que mudaram toda sua rotina e horário de trabalho para irradiar meus animais.

Agradeço à ***equipe do CEMBE,*** pelo auxílio e orientação com os animais. Por me confiarem uma cópia da chave e me permitirem acesso ao vivário mesmo em horário que não estava em funcionamento.

Também a ***todos do Laboratório do Centro de Diagnóstico das Doenças da Boca da UFPEL***, pois me receberam com muito carinho, me ajudaram na confecção de todas as lâminas, e ainda se dispuseram a repeti-las quando foi necessário. À ***Ivana***, por toda disponibilidade e atenção que me deu.

Especialmente à ***professora Ana Paula N. Gomes***, por aceitar o convite de ser minha co-orientadora e abrir as portas para mim na UFPEL, não poderíamos ter feito escolha melhor. Foi um prazer o tempo que estive em Pelotas, mesmo que tenha sido curto. Obrigada por ter se dedicado tanto à minha pesquisa, tua ajuda foi fundamental para a realização de todo o trabalho.

Por fim, agradeço à ***professora Fernanda G. Salum*** por toda orientação dada no decorrer desses dois anos, por me direcionar na realização de toda pesquisa, me conduzindo da melhor forma em cada etapa realizada. Obrigada por dividir comigo seu conhecimento e por toda atenção que deu ao meu trabalho.

RESUMO

RESUMO

A radioterapia direcionada à região de cabeça e pescoço frequentemente envolve as glândulas salivares maiores, as quais sofrem alterações morfológicas e funcionais, resultando em hipossalivação e xerostomia. No primeiro artigo desta dissertação foi realizada uma revisão da literatura com o objetivo de abordar as alterações estruturais observadas nas glândulas salivares e os possíveis mecanismos patogênicos pelos quais o estresse oxidativo, decorrente da radioterapia, causa disfunções salivares. Além disso, foram revisados os métodos de prevenção e regeneração da morfologia acinar e da função glandular. Entre as alterações microscópicas agudas e tardias observadas no tecido glandular irradiado, podem-se citar alterações indicativas de morte celular como a apoptose, hipovascularização, formação de tecido fibroso e edema. Considerando as evidências anteriormente mencionadas, o objetivo deste estudo foi avaliar, em glândulas parótidas de camundongos, o efeito da terapia laser de baixa potência (TLBP) sobre alterações morfológicas causadas pela radioterapia e na imunodetecção da proteína caspase-3. Quarenta e um camundongos *Swiss* foram distribuídos em um grupo controle e três grupos experimentais: radioterapia, laser 2 J e laser 4 J. Os grupos experimentais foram submetidos à radiação ionizante em sessão única de 10 Gy. Nos grupos laser, um laser de diodo, GaAlAs (830 nm, 100 mW, 0,028 cm^2, 3,57 W/cm^2) foi utilizado de forma pontual sobre a região correspondente às glândulas parótidas, com energia de 2 J (20 seg, 71 J/cm^2) ou 4 J (40 seg, 135 J/cm^2) por ponto. Os animais foram eutanasiados 48 h ou sete dias após a radioterapia e as glândulas parótidas dissecadas para análise morfológica e imunodetecção da caspase-3. Não houve diferença significativa entre os grupos na

imunodetecção da caspase-3, entretanto, os grupos laser apresentaram percentuais inferiores aos do grupo radioterapia. Além disso, os resultados indicaram que a TLBP promoveu preservação da estrutura acinar, reduziu a ocorrência de vacuolização citoplasmática e estimulou a vascularização glandular. Entre os protocolos de TLBP, o que utilizou a energia de 4 J apresentou os melhores resultados. Tendo em vista as limitações metodológicas desta pesquisa, mais estudos devem ser conduzidos em animais irradiados, utilizando diferentes protocolos de TLPB e observando a resposta glandular, não apenas em curto prazo, como também em longo prazo, quando a ocorrência de alterações tardias nas glândulas salivares pode ser analisada.

Palavras-chave: Glândulas salivares. Radioterapia. Terapia a laser de baixa intensidade. Caspase-3. Apoptose.

ABSTRACT

ABSTRACT

Head and neck radiotherapy often involves major salivary glands and causes morphologic and functional alterations, resulting in hyposalivation and xerostomia. Literature was reviewed in the first manuscript, addressing the structural changes observed in the salivary glands resulting from oxidative stress caused by radiotherapy and pathogenic mechanisms involved. Preventive and regenerative therapies for altered acinar morphology and glandular function were also discussed. Among the acute and late microscopic alterations observed in glandular tissue, there are particularly changes indicative of cell death, hypovascularization, formation of fibrous tissue and edema. Considering the evidences before mentioned, the aim of this study was to evaluate the effect of low level laser therapy (LLLT) on radiotherapy-induced morphological changes and immunodetection of caspase-3 protein in parotids of mice. Forty-one Swiss mice were divided into a control group and three experimental groups: radiotherapy, 2 J laser and 4 J laser. The experimental groups were exposed to ionizing radiation in a single session of 10 Gy. In the laser groups, a GaAlAs laser (830 nm, 100 mW, 0.028 cm^2, 3.57 W/cm^2) was used on the region corresponding to the parotid glands, with 2 J energy (20 sec, 71 J/cm^2) or 4 J (40 sec, 135 J/cm^2) per point. The animals were euthanized 48 hours or seven days after radiotherapy and parotid glands were dissected for morphological analysis and immunodetection of caspase-3. There was no significant difference between groups in the immunodetection of caspase-3, but the laser groups had a lower percentage compared to the radiotherapy group. Furthermore, the results indicated that LLLT promoted the preservation of acinar structure, reduced the occurrence of cytoplasmic vacuolation and stimulated parotid gland vascularization.

Of the two LLLT protocols, the one using 4 J of energy showed better results. Given the methodological limitations of this study, further researches should be conducted in irradiated animals, using different LLLT protocols and observing glandular response, not only in the short term but also long term, when the occurrence of late changes in the salivary glands can be analyzed.

Keywords: Salivary glands. Radiotherapy. Low level laser therapy. Caspase-3. Apoptosis.

LISTA DE ILUSTRAÇÕES

LISTA DE ILUSTRAÇÕES

RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION: STRUCTURAL CHANGES, PATHOGENETIC MECHANISMS AND THERAPIES

EFFECT OF LOW-LEVEL LASER THERAPY ON IRRADIATED PAROTID GLANDS – A STUDY IN MICE

LISTA DE TABELAS

LISTA DE TABELAS

RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION: STRUCTURAL CHANGES, PATHOGENETIC MECHANISMS AND THERAPIES

EFFECT OF LOW-LEVEL LASER THERAPY ON IRRADIATED PAROTID GLANDS – A STUDY IN MICE

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AIF	Apoptosis-inducing factor
Akt	Protein kinase B
Alda-89	Aldehyde dehydrogenase 3 activator
ALDH3	Aldehyde dehydrogenase 3
Ascl 3	Achaete scute-like 3
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
Gy	Gray
HBO	Hyperbaric oxygenation
IGF-1	Insulin-like growth factor-1
IMRT	Intensity modulated radiotherapy
KGF	Keratinocyte growth factor
LLLT	Low-level laser therapy
MDM2	Murine double minute clone 2
PCNA	Proliferating cell nuclear antigen
PLDR	Potential lethal radiation damage repair
TLBP	Terapia Laser de Baixa Potência

SUMÁRIO

SUMÁRIO

1 INTRODUÇÃO

1 INTRODUÇÃO

A radioterapia consiste na utilização de doses elevadas de radiação ionizante para o tratamento de neoplasias malignas. A radiação interage com os tecidos tumorais, atuando sobre o DNA nuclear por meio da produção de radicais livres, o que leva à morte ou incapacidade de replicação celular. Sua ação sobre os tecidos não é seletiva, atuando também em células saudáveis, o que a torna tóxica para o organismo (1). A radioterapia pode ter indicação terapêutica primária, adjuvante à cirurgia, à quimioterapia ou como método paliativo no manejo de lesões em estágio avançado (2-4). Suas formas de aplicação são a teleterapia, que consiste no emprego da fonte de radiação à distância do tumor e a braquiterapia, onde a fonte se localiza próxima ou no interior do tumor (4,5). A teleterapia é a forma mais comumente utilizada em região de cabeça e pescoço, as doses variam entre 50 e 70 Gy e são fracionadas em 2 Gy ao dia, cinco vezes por semana (3,6). Atualmente, também tem sido utilizada a técnica de radioterapia de intensidade modulada, que preserva estruturas adjacentes ao tumor, uma vez que a dose de radiação mais intensa restringe-se à área do tumor (7).

Apesar de eficaz no tratamento de tumores da região de cabeça e pescoço, a radioterapia pode causar uma série de efeitos adversos tais como mucosite, trismo, osteorradionecrose, xerostomia, dentre outros (8). A xerostomia é resultado da diminuição do fluxo salivar, decorrente da perda de função das glândulas salivares nos pacientes irradiados (8). Alterações salivares quantitativas e qualitativas predispõem tais pacientes a diversas complicações que se desenvolvem direta ou indiretamente, afetando sua qualidade de vida. Dentre estas, cabe citar a perda total

ou parcial do paladar, dor e ardência bucal, suscetibilidade a infecções orais e cáries, disfagia, disfonia e até mesmo alterações psicológicas como a depressão (9).

Apesar de serem estáveis, pois não possuem uma alta taxa mitótica, as células acinares respondem rapidamente à radiação (10-13). A glândula parótida, responsável por aproximadamente 60% da produção de saliva, é apontada como a mais radiossensitiva das glândulas salivares maiores (11,14-16). Entre as alterações agudas e tardias observadas nas glândulas salivares irradiadas estão a perda e atrofia das células acinares, diminuição do peso glandular e formação de tecido fibroso (11, 12, 17,18). Para avaliar a morte celular após a radioterapia, estudos têm analisado a imunodetecção da caspase-3, proteína que exerce um papel importante na apoptose celular (10-12).

Na tentativa de contornar os efeitos adversos da radioterapia sobre as glândulas salivares, estudos realizados em humanos e em modelos animais têm testado diferentes métodos de prevenção e tratamento da xerostomia. Dentre eles é possível destacar os citoprotetores como amifostina, tempol, fatores de crescimento, tratamentos paliativos com saliva artificial, agonistas colinérgicos muscarínicos tais como pilocarpina, cevilemina e betanecol, repovoamento com células-tronco e terapia laser de baixa potência (11, 12,19-24).

De uma forma geral, a terapia laser de baixa potência (TLBP) usa a energia da luz na forma de fótons para produzir respostas celulares (25). Fótons de luz são absorvidos pelos citocromos e porfirinas nas mitocôndrias das células (26,27), ocorrendo liberação temporária de óxido nítrico, o que resulta em aumento da respiração e transcrição celulares (28,29), estímulo à síntese de ATP (trifosfato de adenosina) (30,31) e à formação de espécies reativas de oxigênio, com consequente ativação celular (32). Desta forma, poderá haver ativação de inúmeras vias

intracelulares, regulação da síntese de ácidos nucleicos e de proteínas, modulação dos níveis de citocinas, fatores de crescimento e mediadores inflamatórios, além do estímulo à proliferação e diferenciação celulares. A seleção do comprimento de onda do laser depende, geralmente, do alvo de aplicação e das características ópticas dos componentes teciduais (33). Embora ainda não seja possível determinar o melhor comprimento de onda para cada disfunção, pode-se defini-lo com base no conceito de que o laser vermelho (630 nm a 680 nm) tem profundidade de penetração menor nos tecidos se comparado ao infravermelho (780 nm a 930 nm) (34).

Simões et al. (35) avaliaram a ação da TLBP (808 nm, 500 mW, 277 mW/cm^2, 4J/cm² e 8J/cm²) em glândulas salivares maiores de ratos. Foram realizadas duas sessões de tratamento em dois dias consecutivos e a saliva foi coletada em três momentos: imediatamente após cada sessão e uma semana após o início da TLBP. Foi observado aumento no fluxo salivar na terceira coleta de saliva em comparação à primeira. Simões et al. (23) avaliaram também a resposta das glândulas salivares à TLBP (660 nm, 40 mW, 0,036 cm^2, 6 J/cm²) em 22 pacientes que estavam sendo submetidos à radioterapia ou que já haviam finalizado este tratamento. Os pacientes foram distribuídos em dois grupos os quais receberam uma ou três sessões de TLBP por semana. O fluxo salivar foi mensurado antes e após cada sessão. Os resultados demonstraram que houve aumento significativo no fluxo salivar dos pacientes que já haviam finalizado a radioterapia, sem diferença entre o grupo que recebeu TLBP uma vez por semana e o grupo submetido a três sessões semanais. Naqueles pacientes que ainda estavam sob tratamento radioterápico, a realização de três sessões semanais de TLBP impediu que houvesse redução significativa no fluxo salivar.

Loncar et al. (36) investigaram o efeito da TLBP (904 nm, 6 mW, 4,44 mm^2, 246 mW/cm^2, 29,5 J/cm^2) em 34 pacientes com xerostomia. Os indivíduos receberam a terapia nas glândulas parótidas, submandibulares e sublinguais durante 10 dias consecutivos. No grupo-controle foi administrado ácido cítrico. A quantidade de saliva foi mensurada antes e 5 minutos após a utilização do laser e do ácido cítrico durante os 10 dias de tratamento. A quantidade de saliva produzida no grupo-laser aumentou linearmente no decorrer da pesquisa, enquanto no grupo-controle houve aumento do fluxo salivar na primeira metade do estudo, com um declínio nas coletas seguintes.

Considerando-se as alterações das glândulas salivares decorrentes da radioterapia e que a TLBP tem sido utilizada no tratamento da xerostomia e da hipossalivação, o objetivo deste estudo foi avaliar seu efeito sobre alterações morfológicas radioinduzidas e na imunodetecção da caspase-3 em parótidas de camundongos.

2 PROPOSIÇÃO

2 PROPOSIÇÃO

2.1 Objetivo Geral

Avaliar o efeito da terapia laser de baixa potência (TLBP) sobre alterações morfológicas induzidas pela radioterapia e sobre a apoptose das células acinares de glândulas parótidas de camundongos.

2.2 Objetivos Específicos

- Realizar uma revisão da literatura abordando as alterações estruturais decorrentes da radioterapia nas glândulas salivares, os mecanismos patogênicos envolvidos, bem como as terapias preventivas e regenerativas para tais alterações.
- Avaliar o efeito da TLBP sobre alterações morfológicas agudas decorrentes da radioterapia em glândulas parótidas de camundongos.
- Investigar o efeito da TLBP sobre a imunodetecção da proteína caspase-3 em glândulas parótidas de camundongos irradiados.
- Verificar o efeito de dois protocolos de TLBP em glândulas parótidas de camundongos submetidos à radioterapia.

3 ARTIGO DE REVISÃO DA LITERATURA

3 ARTIGO DE REVISÃO DA LITERATURA

RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION: STRUCTURAL CHANGES, PATHOGENETIC MECHANISMS AND THERAPIES

Artigo submetido para avaliação (Anexo C)

Periódico: Archives of Oral Biology

Qualis Capes Odontologia 2013: A2

Fator de Impacto: 1,549

RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION: STRUCTURAL CHANGES, PATHOGENETIC MECHANISMS AND THERAPIES

RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION

ACAUAN*, Monique Dossena

FIGUEIREDO*, Maria Antônia Zancanaro

CHERUBINI*, Karen

GOMES**, Ana Paula Neutziling

SALUM*, Fernanda Gonçalves

***Oral Medicine Division, Pontifical Catholic University of Rio Grande do Sul- PUCRS, Brazil.**

****Oral Pathology Division, Federal University of Pelotas – UFPEL, Brazil.**

Corresponding address:
Fernanda Gonçalves Salum
Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS
Hospital São Lucas
Av. Ipiranga, 6690 – Room 231
CEP: 90610-000 - Porto Alegre – RS – Brazil
Tel/Fax: +55 51 3320-3254
E-mail: fernanda.salum@pucrs.br

ABSTRACT

Purpose: This review addressed the structural changes observed in salivary glands and pathogenic mechanisms resulting from oxidative stress caused by radiotherapy. The preventive and regenerative therapies for altered acinar morphology and glandular function were also reviewed. Among acute and late microscopic alterations in glandular tissue, there are particularly changes indicative of cell death, hypovascularization, formation of fibrous tissue and edema. A critical role was identified for the Akt–MDM2–p53 pathway in the suppression of DNA damage-induced apoptosis in acinar cells. Prophylactic treatment with pilocarpine, cevilemine, bethanechol and isoproterenol has shown a positive effect on salivary flow, but lasting results have not been observed. The administration of growth factors, besides histamine and lidocaine, has also demonstrated radioprotective effects on the salivary glands. Stem cell preservation and transplantation may be an alternative to maintain tissue homeostasis and thus allow glandular regeneration. Conclusion: Knowledge of the structural changes observed in the salivary glands contributes to proving the short- and long-term efficacy of the therapies investigated. It is important to know the molecular mechanisms involved in radiation-induced damage, since the control of the pathogenic mechanisms can inhibit the initial process of tissue degeneration. The challenge for investigators is to protect normal cells selectively, without promoting tumor growth.

Keywords: Salivary gland. Radiotherapy. Structural changes. Therapies.

1 INTRODUCTION

Although effective in treating malignant neoplasms of the head and neck, radiotherapy has side effects, where its action in tissues is not selective, affecting normal cells as well as tumors. The major salivary glands are often irradiated as they are close to the sites of primary tumors and lymph nodes[1,2]. Approximately 70% of patients receiving head and neck radiotherapy develop hyposalivation due to a progressive loss of salivary gland function[2]. The hyposalivation can be observed during the first weeks of treatment and often persists throughout the patient's life[1,3]. Due to the quantitative and qualitative changes that occur in saliva, patients become vulnerable to complications that directly or indirectly affect their quality of life. These complications include loss of taste, painful and burning mouth, susceptibility to caries and other oral infectious diseases, dysphagia, dysphonia and even psychological disorders such as depression[4].

There are no studies that clearly show how radiotherapy acts on the function of the salivary glands. Although acinar cells do not have a high mitotic activity, they show an early response to radiation[5-8]. Disrupted signal transduction as a result of radiation-induced damage to the plasma membrane has been suggested to be the cause of the decrease in salivary flow observed just after radiation[9]. However, this effect does not explain the persistence of hyposalivation for years. Macroscopic and microscopic changes in salivary glands resulting from radiation are also described in the literature; many studies indicate that there is a close relation between acinar loss and chronic glandular dysfunction[5,10-12].

Aiming to improve the quality of life of patients treated with radiotherapy, investigators have sought to develop treatments that reduce the effect of ionizing radiation on the salivary glands. However, many treatments produce a short-term

improvement in salivary flow but have no effect on acinar morphology[6]. The aim of this review was to address the structural changes observed in the salivary glands resulting from oxidative stress caused by radiotherapy and to describe the pathogenic mechanisms involved. Preventive and regenerative therapies for altered acinar morphology and glandular function are also discussed.

2 INFLUENCE OF RADIATION DOSE ON THE FUNCTION AND RECOVERY OF SALIVARY GLANDS

Different levels of hyposalivation have been observed in patients after completion of radiotherapy. The area of salivary tissue exposed and the dose of radiation are the main factors that influence the glandular changes as shown by some studies[13-15]. Buus et al.[16] found a direct relationship between the decline in glandular function and increased radiation dose. Li et al.[14] and Eisbrush et al.[17] reported that the recovery of salivary flow can occur with doses up to 24 and 26 Gy, respectively. On the other hand, Murdoch-Kinch et al.[15] conducted a similar study evaluating salivary flow in submandibular glands and found that the recovery of salivary flow occurs at radiation doses up to 39 Gy. On average, recovery of gland function occurs within two years after the end of radiotherapy[14,15].

To limit the adverse effects of radiation therapy, intensity-modulated radiotherapy (IMRT) has been developed. This therapy focuses on a higher dose of radiation in the tumor tissue. However, in many cases of head and neck malignant neoplasms, a high dose of radiation still reaches the adjacent tissues, including the salivary glands, which are exposed to doses higher than 30 Gy[18].

3 MACROSCOPIC GLANDULAR CHANGES

Studies report a macroscopically detectable loss of the structure of the salivary glands as a consequence of radiotherapy[19-22]. Ricchetti et al.[22] measured the volume of the parotid and submandibular glands during radiotherapy and found that reduction in glandular size was significant in the first week. Fiorentino et al.[19] observed a linear decrease in the volume of the parotid glands in patients undergoing IMRT. Radiation doses ranged from 24.9 to 37 Gy; and on the twentieth day of treatment, the glands had lost about 30% of their original volume.

Nagler et al.[20] irradiated mice with doses between 2.5 and 15 Gy and found a decrease in weight of the parotid and submandibular glands. This decrease was proportional to radiation dose; weight of the parotid and submandibular glands decreased to 60 and 40% of the initial value, respectively. The decrease in the volume of parotid and submandibular glands of minipigs irradiated with 70 Gy also varied between 50 and 60% compared to the control group. Histological analysis confirmed the presence of acinar atrophy[21].

4 MICROSCOPIC GLANDULAR CHANGES

Several studies have investigated the effect of radiotherapy on salivary gland morphology[6,7,23-26]. Among the acute and late microscopic alterations observed in glandular tissue, there are particularly changes indicative of cell death, hypovascularization, formation of fibrous tissue and edema (Table 1).

Table 1. Macroscopic and microscopic changes evaluated in salivary glands of irradiated animals.

Authors	Models	Glandular weight/size reduction	Fibrosis	Vascular changes	Edema	Cytoplasmic vacuolation	Nuclear changes	Loss of acinar cells
Stephens et al.[25]	Rehsus Monkey	+	+	+	+	+	+	+
Henriksson et al.[24]	Rat	°	+	°	+	°	°	+
Coopes et al.[6]	Rat	°	+	°	+	+	+	+
Coopes et al. [27]	Rat	+	+	°	°	°	°	+
Friedrich et al.[44]	Rat	+	+	°	°	°	°	°
Radfar and Sirois[21]	Minipig	+	+	°	°	+	+	+
Hakim et al.[23]	Rat	°	°	+	°	+	+	+
Lombaert et al.[61]	Mouse	°	+	°	°	°	°	+
Limesand et al.[11]	Mouse	°	+	°	°	+	°	+
Teymoortash et al.[84]	Rat	+	+	°	°	°	°	+
Limesand et al.[28]	Mouse	°	+	°	°	°	+	+
Xu et al.[26]	Minipig	°	+	+	°	°	°	+
Hakim et al.[47]	Rat	°	+	°	°	°	+	°
Nanduri et al.[62]	Mouse	°	+	+	°	°	°	+
Xiang et al.[72]	Rat	°	°	°	°	+	+	+

(+) Tissue changes found in salivary glands.
(°) Alterations not found or not reported by the authors.

4.1 Cell Death

The loss of acinar cells resulting from radiotherapy is reported in several studies[5,12,21,23-28]. However, cell death is evident not only by the decrease in the number of acinar cells in irradiated tissues[12,27] but also by the presence of apoptotic cells and cytoplasmic vacuolation, as well as other nuclear and cytoplasmic changes.

4.1.1 Apoptotic cells

The increase in apoptotic cell number has been regarded as a major cause of the dysfunction of the salivary glands resulting from radiotherapy[5-7,9,12]. Apoptosis is a rapidly occurring phenomenon, identified morphologically by cell shrinkage and condensation of chromatin, which localizes next to the nuclear membrane. Subsequently, apoptotic bodies are formed, which are phagocytosed by

macrophages without triggering an inflammatory reaction[29]. The activation of apoptosis occurs by the extrinsic (cytoplasmic) or intrinsic (mitochondrial) pathway[30]. Apoptosis appears to involve changes in mitochondria when resulting from ionizing radiation[30,31]. Apoptotic signals such as DNA damage, deprivation of growth factors and hypoxia cause changes in mitochondrial membrane permeability and the release of cytochrome C to the cytoplasm. As a consequence, caspase-9 is released, which activates caspase-3[32]. There is also the loss of cellular homeostasis, production of reactive oxygen species (ROS) and interruption of ATP synthesis. High levels of ROS further enhance mitochondrial membrane permeability and activation of caspase-9 and -3[33]. The involvement of apoptosis-inducing factor (AIF) is also described in the literature, which acts independently of caspases, after its activation it is translocated to the nucleus causing chromatin condensation and DNA fragmentation[34].

To check the number of apoptotic cells in salivary glands undergoing radiotherapy, studies have evaluated the expression of caspase-3 protein[5,7,28,35]. Increased levels of the protein can be seen during the first hours after radiation[5,7,28]. Recently, investigations have indicated that apoptosis of epithelial cells may contribute to loss of stem cells[36], which are found in the salivary gland ductal compartment[12].

4.1.2 Cytoplasmic vacuolation

Microscopically, vacuolation is characterized by clear areas that can be spherical or oval and varying in size[37]. The vacuoles in the cytoplasm represent an active process of autophagy[28]. This process is induced under shortage of nutrients,

infection and oxidative stress, in which the cells need to generate intracellular nutrients and energy and to get rid of damaging cytoplasmic components[38].

Several authors have described the presence of cytoplasmic vacuolation in salivary glands of irradiated animals[6,23,25,28]. Stephens et al.[25] found vacuolated cells up to 72 hours after radiation in the glands of monkeys irradiated with doses of 12.5 and 15 Gy. An increased number of vacuolated acinar cells were also observed by Coopes et al.[6]. The vacuolation regresses between 72 hours and one month after radiotherapy[23].

4.1.3 Nuclear and cytoplasmic changes

Nuclear and cytoplasmic changes such as increased cell volume, chromatin condensation, cytoplasmic disorganization and loss of plasma membrane integrity are characteristic of cell necrosis, a process of passive cell death resulting from damage. In this process, after cell disruption, intracellular contents are released, generating a local inflammatory response[29].

Coopes et al.[6] have described nuclear changes observed in salivary glands of irradiated rats as aberrant nuclei; the number of abnormal cells was approximately 3%. Hakim et al.[23] reported anisonucleosis and rupture of the cell membrane between 72 hours and thirty days after irradiation. Limesand et al.[28] irradiated mice and observed a nuclear enlargement of acinar cells between 24 and 96 hours after radiation with fractionated doses of 2 Gy/day, between one and five days.

4.2 Hypovascularization

Studies on salivary glands of irradiated animals have shown changes in blood flow and distribution of blood vessels; these changes are seen as factors responsible for tissue damage[39,40]. Xu et al.[26] irradiated minipigs with a dose of 25 Gy, and after four hours, they found more than a 40% decline in blood flow of parotid glands. Subsequently, the flow remained 20% lower than in non-irradiated glands. The density of glandular microvessels was reduced by approximately 25% 24 hours after radiation therapy, and 36% after two weeks. A significant increase in the number of apoptotic endothelial cells was also observed.

The influence of vascular changes on acinar atrophy may be due to lower potential of regeneration and cell survival caused by hypoxia and hypovascularization[26,41,42].

4.3 Fibrosis

Fibrosis is characterized by excessive collagen, glycosaminoglycans and other extracellular matrix components. Radiotherapy is a mediator of fibrosis, resulting from inflammation, injury and cell death[42,43]. Microvascular injury, cited above, also promotes an initial stimulus for fibrosis by tissue hypoxia[42].

Fibrous tissue formation in healthy organs that lie within the field of ionizing radiation is responsible for the loss of tissue function[42] and represents one of the chronic glandular changes after radiotherapy[6,21,44]. Hakim et al.[45] studied changes in the parotid and submandibular glands of humans irradiated with 60-72 Gy. Distorted

arrangements of acinar cells dispersed in widely distributed fibrous tissue were observed. This change was seen in the first ten days after radiotherapy.

The fibrosis formation depends on the total dosage and the position of the gland related to the radiation source[42]. Henriksson et al.[24] observed in the parotid and submandibular glands of rats that increased mast cell density in the tissue was radiation dose-dependent. This increase was associated with the reduction in acinar cell number and concomitant fibrosis.

4.4 Edema

Edema is the abnormal accumulation of fluid in the interstitial extracellular compartment or in body cavities. Increased net content was detected, indicating high water level in the intravascular and extracellular space in parotid and submandibular glands of patients six months after radiation. Despite the edema, a 25% decrease in glandular volume was observed. Furthermore, a relation between location receiving a higher dose of radiation and extent of edema was suggested[46].

Coopes et al.[6] observed in the parotid glands of mice that the contact area between acini was reduced, suggesting interstitial edema 10 days after irradiation with 15 Gy. Henriksson et al.[24] also showed signs of edema in the glandular parenchyma due to the inflammatory reaction caused by radiotherapy in mice.

5 MOLECULAR MECHANISMS, PREVENTIVE THERAPY AND REPAIR OF RADIATION-INDUCED SALIVARY GLANDS DAMAGE

To preserve long-term glandular function in irradiated patients, authors have studied radiation-induced molecular alterations (Figure 1), protective therapy and repair of salivary glands, such as the use of stem cells, protective drugs, hyperbaric oxygen and botulinum toxin[6,23,47-49].

5.1 Molecular mechanisms and stem cells

5.1.1 p53

Radiation-induced apoptosis seems to be mediated through a p53-dependent pathway[5,7]. DNA damage leads to p53 transcriptional activation, resulting in cell cycle arrest and the activation of proapoptotic genes such as Bax and PUMA[50].

Avila et al.[5] irradiated genetically engineered mice with a deletion of the p53 gene and studied the effect on apoptosis by determining caspase-3 expression. Animals were irradiated with 1, 2, 5 and 10 Gy, and the parotid glands were assessed 24 hours later. The incidence of apoptotic cells in p53+/- and p53+/+ animals was dependent on the radiation dose; the expression of caspase-3 in p53 +/+ animals was significantly higher than in animals p53 +/- with 10 Gy radiation. In contrast, in p53-/- mice, no radiation-induced apoptosis was observed. Furthermore, the salivary flow was measured to determine the influence of apoptosis on glandular function. While the p53 +/- and p53 +/+ groups showed a significant decrease in salivary flow, the p53-/- group showed no reduced flow.

Studies have shown that p53 also regulates cellular autophagy and senescence. Both p53 activation and inhibition can induce autophagy[10,51]. Radiation-induced DNA damage can result in p53-dependent senescence[52].

5.1.2 Protein kinase B (Akt)

In salivary acinar cells, the expression of Akt leads to the phosphorylation of murine double minute clone 2 (MDM2), which inhibits p53 transcriptional activation and, thus, DNA damage–induced apoptosis[7]. Limesand et al.[7] observed a decrease in apoptotic cells 24 hours after 5 Gy irradiation in transgenic mice expressing a constitutively activated mutant of Akt1 (myr-Akt1) compared with wild-type. The acinar cells of Akt mutant mice exposed to radiation doses of 0.25 to 5 Gy also showed resistance to radiation compared to wild-type mouse cells[7].

5.1.3 Insulin-like growth factor-1 (IGF-1)

Limesand et al.[11] demonstrated that IGF-1 stimulates endogenous Akt activation in salivary glands. IGF-1 was administered intravenously in mice and Akt activation in parotid glands was determined by immunoblotting. A high level of Akt activation was observed 5 minutes after the administration of 5 mg IGF-1. Akt activation remained at high levels for 4 hours. IGF-1 was also evaluated in irradiated salivary glands. Twenty-four hours after radiotherapy, animals treated with IGF-1 showed 4% apoptotic cells, a significantly lower rate compared to the control group, which showed 13% apoptotic cells. Salivary flow was measured three and 30 days after radiotherapy, with no decrease in comparison to the non-irradiated group.

Grundmann et al.[53] administered IGF-1 to mice for five consecutive days and the first application was on the fourth day after 5 Gy irradiation. After thirty days, salivary flow in animals treated with IGF-1 was 72% compared to the initial value and significantly higher than in the animals only irradiated. In the same study, the area of functional acinar cells in parotid glands was quantified, and a regeneration of glandular structure was seen in the IGF-1-treated animals.

Limesand et al.[28] evaluated the salivary glands of mice irradiated with daily fractionated doses of 2 Gy for five consecutive days, which received IGF-1 injections immediately before radiotherapy sessions. Apoptotic cells increased significantly, especially in the first 24 hours after each irradiation. In contrast, IGF-1-treated animals showed a significant decrease in apoptosis. In agreement with other studies[5,11], over 95% of apoptotic cells were acinar cells. Parotid gland sections were evaluated for structural abnormalities 90 days after radiotherapy. In all radiation-treated animals, there was evidence of atrophy, fibrosis, or sclerosis, and there were acinar cells containing enlarged nuclei and areas of dispersed inflammatory cells. In IGF-1-treated animals, no histological changes were detected. In addition, PCNA (proliferating cell nuclear antigen) expression was increased in these animals.

5.1.4 Basic fibroblast growth factor (bFGF)

bFGF is a physiological agent characterized as an inducer of potentially lethal radiation damage repair[54-56]. It induces cells to undergo an extended G2 arrest after irradiation, allowing more time for the cells to recover from DNA damage prior to mitosis, thereby enhancing clonogenic survival[54].

Thula et al.[57] investigated the radioprotective effect of bFGF on parotid acinar cells of rats. Organ cultures were incubated in bFGF-supplemented media 4 hours prior and immediately after 15 Gy irradiation. Administration of bFGF partially protected the parotid gland, reducing the increase in the rate of apoptosis by 44%.

5.1.5 Keratinocyte growth factor (KGF)

Several studies have suggested that KGF can increase the radioresistance of epithelial cells by enhancing DNA repair[58], by altering the expression of mediators or antagonists of apoptosis[59], or by altering the ability of cells to scavenge free radicals[60].

Lombaert et al.[12] demonstrated the efficacy of KGF to protect submandibular glands of mice irradiated with a single dose of 15 Gy. Recombinant KGF was administered before and after radiation; saliva production and weight gland were preserved. Massive depletion of acinar cells and deposition of fibrotic cells were clearly visible 90 days after irradiation. In contrast, KGF almost completely abrogated the net loss of acinar cells. In the same study, pretreatment of cultured cells with KGF increased stem cell survival after irradiation and accelerated the proliferation of these progenitor cells.

5.1.6 Stem cell transplantation

In the long term, stem cell preservation may be an alternative to maintain tissue homeostasis and thus allow glandular regeneration. Studies have reported that

ductal cells are capable of differentiating into acinar cells in culture, indicating the presence of stem cells[10,61,62].

Lombaert et al.[61] tested the ability of irradiated submandibular glands to produce saliva from an injection of cells obtained from the submandibular glands of mice. Mice were irradiated and cells transplanted 30 days later. Ninety days later, ductal structures were formed at the injection site. Transplanted glands were similar in morphology to non-irradiated glands and a large number of acinar cells were seen. Furthermore, a significant increase in salivary flow was observed in 42% of animals. In this study, the authors also isolated human salivary gland cells. The authors observed that human cells showed the same behavior as mouse cells, where stem cells were also detected in ductal compartments and, as in mouse cells, expressed the c- kit gene.

Nanduri et al.[62] cultivated c-kit cells from salivary glands of mice. Mice were irradiated with a single dose of 15 Gy, and after 30 days, cells were transplanted. Ninety days later, salivary flow increased approximately 40% when compared to the non-transplanted group. In transplanted animals, the number of acinar cells increased, innervation and vascularization were preserved, and the formation of fibrous tissue was prevented. Also, the presence of stem cells in ductal compartments was observed in transplanted animals, and it was undetectable in irradiated and non-transplanted animals. This may indicate a potential for tissue recovery in the long term.

Feng et al.[10] investigated the presence and *in vitro* potential of human salivary gland stem cells. Although human and mouse salivary glands were not exactly the same, the tissue architecture after irradiation looked remarkably similar. In both species, the ductal compartment necessary for stem cell engraftment largely

remained intact; moreover, the formation of salispheres in human salivary gland cells was very similar to that in mice. These results indicate that human salispheres do contain cells with stem cell-like properties. Furthermore, these cells could be isolated from human salispheres in substantial numbers, albeit in lower percentages than from rodent salispheres. Authors attribute this to a lower stem cell number in older people such as patients with head and neck cancer.

5.1.7 Aldehyde dehydrogenase 3 activator (Alda-89)

Both adult human and murine stem cells express higher levels of ALDH3 isozymes compared to other cells[63]. According to Banh et al.[63], activating ALDH3 with Alda-89 enhances salivary stem cell survival and proliferation *in vivo*.

To preserve the survival of submandibular gland stem cells and to increase their proliferation after radiotherapy, Xiao et al.[64] used an osmotic pump containing 3.4 mol/L Alda-89 and implanted it intraperitoneally in mice. It was demonstrated that Alda-89 prevented a decrease in salivary flow eight weeks after radiotherapy. In histological evaluation, acinar structures were better preserved in mice treated with Alda-89. The percentage of total acinar area was 51% compared to 26 % in untreated mice. The total area of acini in non-irradiated animals covered 60-70% of the glands. Furthermore, the study demonstrated that Alda -89 does not protect cancer cells in culture or promote tumor growth *in vivo* and is not toxic.

5.1.8 Wnt signaling pathway

The intracellular signaling pathway Wnt/β-catenin plays an essential role in the differentiation, proliferation, death, and function of various cell types[65]. Its activity is increased in progenitor cells and forced activation improves the tissue regeneration process. When the pathway is inhibited, the regeneration process is impaired[66,67].

Hai et al.[36] evaluated the effects of radiation on Wnt activity in salivary glands. Wnt reporter transgenic mice were exposed to 15 Gy of single-dose radiation in the head and neck area. Transient Wnt1 overexpression in basal epithelia was induced in inducible Wnt1 transgenic mice before, together with, after, or without local radiation, and saliva flow rate, histology, apoptosis, proliferation, stem cell activity, and mRNA expression were then evaluated. Concurrent transient activation of the Wnt pathway prevented a decrease in salivary flow 30, 60 and 90 days after radiotherapy. Authors observed a significant inhibition of apoptosis and BAX and PUMA expression and an increase in survivin expression compared to only irradiated animals. Ninety days after radiation, PCNA and Ascl3 (achaete scute-like 3 – stem cell proliferative activity marker) expression was also increased; in the control group, however, the expression of these markers was reduced. The authors suggest that the activation of the Wnt pathway may influence tissue homeostasis after radiotherapy by increasing the active progenitor cells and preventing a chronic loss of tissue function.

Hakim et al.[45] studied the expression of Wnt/β-catenin in human salivary glands, which were harvested from patients previously irradiated for head and neck cancer. The radiotherapy dose in 2-Gy fractions ranged from 60 to 72 Gy. Considering irradiated but viable acinar structures, Wnt-1 expression increased along

with the membrane upregulation of β-catenin. These results demonstrated that activation of the Wnt pathway provides a key radioprotective mechanism in irradiated cells.

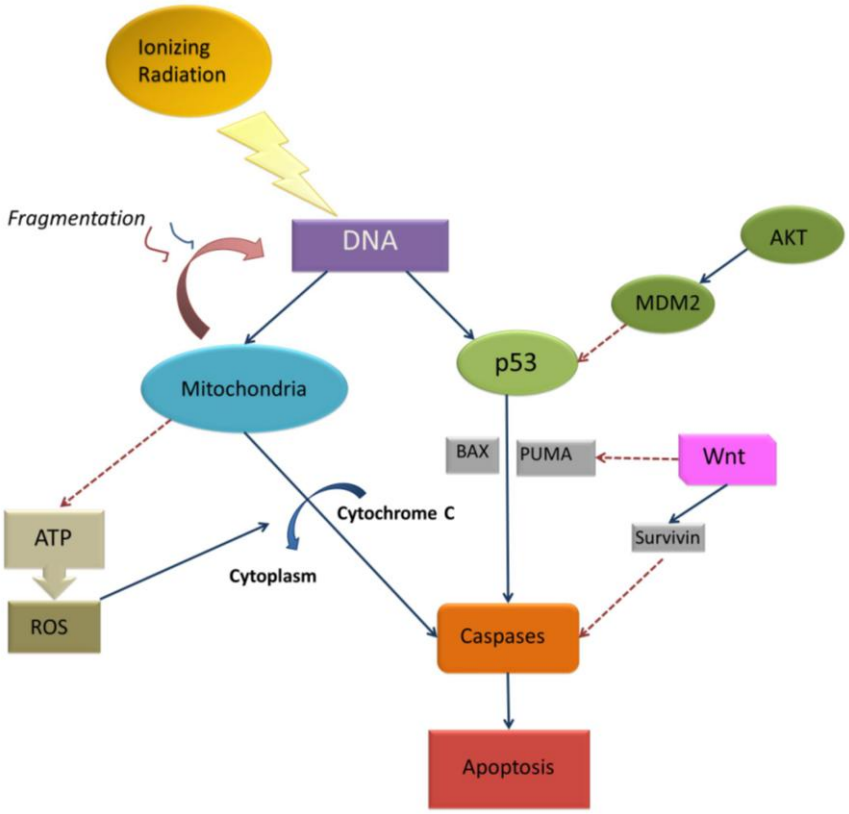

Figure 1 Representation of molecular mechanisms with activations/inductions (arrow, full line) or inhibitions (arrow, dotted line) caused by radiotherapy. ROS (reactive oxygen species), Akt (protein kinase B), MDM2 (murine double minute clone 2).

5.2 Radioprotective drugs

5.2.1 Muscarinic cholinergic and adrenergic agonists

Prophylactic treatment with drugs such as pilocarpine, cevilemine, bethanechol and isoproterenol have shown a positive effect on salivary flow in animals and

humans within 30 days after radiotherapy, but more lasting results have not been observed[68-70].

Coopes et al.[6] investigated the late effects of muscarinic and/or adrenergic receptor agonists on the parotid glands. Mice pretreated with phenylephrine, isoproterenol, methacholine, pilocarpine or methacholine associated with phenylephrine were irradiated with a single dose of 15 Gy. Methacholine administered with phenylephrine showed better results in salivary flow preservation compared to the other drugs administered alone. One month after radiotherapy, this drug combination preserved the acinar cells and showed a lower amount of aberrant nuclei, indicating delayed cell death. At day 120, acinar cell number remained significantly higher than in non-pretreated irradiated rats only in the methacholine plus phenylephrine pretreated group, whereas the number of aberrant nuclei was similar. At day 240 after irradiation, the methacholine plus phenylephrine pretreated group displayed less fibrosis and a better but not normal structure of the acini, compared to the non-pretreated group. The other drugs had no effect in preserving the morphological structure of the salivary glands, except phenylephrine, which showed some protection.

Xiang et al.[71] found that phenylephrine could reduce DNA fragmentation, downregulate the expression of Bax, and inhibit the activation of caspase-3 due to oxidative stress caused by ischemia/reperfusion during autotransplantation of submandibular glands of rats in treatment for severe keratoconjunctivitis sicca. Xiang et al.[72] irradiated rats with a dose of 20 Gy thirty minutes after injection of phenylephrine. Glands of rats receiving the drug remained similar to those of non-irradiated animals. In animals not treated with phenylephrine, vacuolation and pyknotic nuclei were observed in acinar cells. In treated glands, PCNA expression

increased and the level of atrophy as well as the number of apoptotic cells was lower when compared to the untreated control group.

5.2.2 Histamine

Histamine is a biogenic amine that can modulate water secretion in saliva produced by submandibular glands[73]. A radioprotective effect was observed for normal cells in submandibular salivary glands of rats, suggesting histamine may act against free radicals in these cells[74,75]. In addition, histamine can increase the radiosensitivity of malignant cells and exerts different effects on biological responses of normal and cancer cells[73-75]. Histamine administration showed no local or systemic side effects in rats[74].

Medina et al.[75] treated mice with subcutaneous histamine injection 24 hours prior to 5 Gy single-dose irradiation. The treatment prevented a decrease in salivary flow and glandular weight and preserved glandular structure, and also caused a decrease in apoptosis. Moreover, a decrease in Bax protein expression and an increase in PCNA expression were observed in histamine-treated animals.

5.2.3 Lidocaine

Studies have reported the capability of local anesthetics to stabilize and protect the plasma membrane during radiation in cell cultures[76]. Hakim et al.[23] injected lidocaine into rats before exposing the animals to 15 Gy of radiation. Lidocaine prevented salivary flow reduction and preserved parotid gland structure. Also, the drug reduced tenascin C expression and prevented a decrease in smooth muscle

actin detection. Non-treated animals showed intracellular edema, cytoplasmic organelles reduction and vacuolation, while lidocaine-treated animals showed normal morphology.

Hakim et al.[47] compared two protocols of lidocaine administration to preserve salivary flow and morphology of the submandibular and parotid glands of irradiated rats. Before each irradiation session, 10 or 12 mg/kg lidocaine were injected intravenously. The results showed salivary flow preservation in both groups, but only the 12 mg/kg dose was significantly different than the control group. In animals not treated with lidocaine, nuclear and mitochondrial changes were observed in acinar cells. Lidocaine-treated animals displayed glandular morphology similar to that of non-irradiated animals, regardless of the dose administered.

5.3 Hyperbaric oxygen therapy

The use of hyperbaric oxygenation (HBO) to stimulate tissue healing is based on the premise that increased oxygen pressure in tissues in the short term produces an anti-inflammatory effect, vasoconstriction, edema reduction and phagocytosis activation. In the long term, HBO results in angiogenesis, stimulation of collagen synthesis and activation of stem cells[77-80].

Few studies have investigated the effect of HBO on irradiated salivary glands. Williamson[49] examined microscopically the salivary glands of irradiated rats. After radiation, HBO was performed for four weeks. Acinar structure in irradiated glands treated with HBO was similar to that of non-irradiated glands 36 weeks after the end of the experiment; moreover, in irradiated and non-treated glands, less than 50% of acini were observed.

5.4 Botulinum toxin

Botulinum toxin has been used in patients with sialorrhea for hypersalivation treatment. Teymoortash et al.[81] observed in rats a significant decrease in the secretory granules in acinar cells of botulinum toxin-treated submandibular glands. Studies indicate a number of secretory granules as an important pathogenic factor for gland destruction during radiation therapy, since these granules contain large amounts of heavy metals, particularly iron and copper, which could increase sensitivity to ionizing radiation[82,83].

To assess the effect of botulinum toxin on morphological changes in irradiated salivary tissue, Teymoortash et al.[84] injected the drug in the submandibular glands of rats, unilaterally, and comparison was made with the contralateral gland. After treatment, animals were irradiated and the glands were assessed by scintigraphy and morphologically analyzed after 90 days. Significant reduction in the volume and weight of the untreated glands was observed, as well as periductal and parenchymal fibrosis with destruction of lobular architecture. The authors reported slight changes in these structures in glands treated with botulinum toxin. A higher percentage of cells with fragmented DNA (subG1), representing dead cells, was observed in control glands, whereas a lower percentage of subG1 population was identified in glands pretreated with botulinum toxin after radiotherapy.

6 CONCLUSIONS

A better understanding of glandular response against radiotherapy-induced oxidative stress is essential for the development of preventive and therapeutic

measures for radiation damage. Knowledge of the structural changes observed in the salivary glands contributes to determining the short and long term efficacy of the therapies investigated. Acinar cells show early response to radiation; among acute and late microscopic alterations in glandular tissue, there are particularly changes indicative of cell death, hypovascularization, formation of fibrous tissue and edema. Besides these structural changes, studies have also investigated the molecular mechanisms involved in radiation-induced damage, since the control of the pathogenic mechanisms can inhibit the initial process of tissue degeneration. A p53-dependent pathway appears to mediate radiation-induced apoptosis; DNA damage leads to p53 transcriptional activation, resulting in cell cycle arrest and the activation of proapoptotic genes such as Bax and PUMA. Furthermore, studies have shown that phosphorylation of MDM2 by Akt leads to p53 inactivation. The administration of growth factors such as IGF-1, *bFGF* and *KGF*, besides histamine and lidocaine, has demonstrated radioprotective effects in salivary glands. However, the challenge for investigators is to be able to protect normal cells selectively without promoting tumor growth. Studies that focus on the protection of stem cells for later tissue regeneration seem to be promising in light of the good results already achieved.

REFERENCES

1. Bhide SA, Ahmed M, Newbold K, Harrington KJ, Nutting CM. The role of intensity modulated radiotherapy in advanced oral cavity carcinoma. J Cancer Res Ther 2012; 8:67-71.
2. Mossman K, Shatzman A, Chencharick J. Long- term effects of radiotherapy on taste and salivary function in man. Int J Radiat Oncol Biol Phys 1982;8:991-7.
3. Epstein JB, Thariat J, Bensadoun RJ, Barasch A, Murphy BA, Kolnick L, et al. Oral complications of cancer and cancer therapy. CA Cancer J Clin 2012;62:400–22.
4. Dirix P, Nuyts S, Poorten VV, Delaere P, van den Bogaert W. The influence of xerostomia after radiotherapy on quality of life. Support Care Cancer 2008;16:171-9.
5. Avila JL, Grundmann O, Burd R, Limesand KH. Radiation-induced salivary gland dysfunction results from p53-dependent apoptosis. Int J Radiat Oncol Biol Phys 2009;73:523–9.
6. Coopes RP, Zeilstra LJW, Kampinga HH, Konings AWT. Early to late sparing of radiation damage to the parotid gland by adrenergic and muscarinic receptor agonists. B J Cancer 2001;85:1055-63.
7. Limesand KH, Schwertfeger KL, Anderson SM. MDM2 is required for suppression of apoptosis by activated Akt1 in salivary acinar cells. Mol Cell Biol 2006;26:8840–56.
8. Paardekooper GM, Cammelli S, Zeilstra LJ, Coopes RP, Konings AW. Radiation-induced apoptosis in relation to acute impairment of rat salivary gland function. Int J Radiat Biol 1998;73:641-8.

9. Konings AW, Coopes RP, Vissink A. On the mechanism of salivary gland radiosensitivity. Int J Radiat Oncol Biol Phys 2005;62:1187-94.
10. Feng J, van der Zwaag M, Stokman MA, van Os R, Coopes RP. Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation. Radiother Oncol 2009;92:466–71.
11. Limesand KH, Said S, Anderson SM. Suppression of radiation-induced salivary gland dysfunction by IGF-1. PLoS One 2009;4:e4663.
12. Lombaert IM, Brunsting JF, Wierenga PK, Kampinga HH, de Haan G, Coopes RP. Keratinocyte growth factor prevents radiation damage to salivary glands by expansion of the stem/progenitor pool. Stem Cells 2008; 26:2595–601.
13. Deasy JO, Moiseenko V, Marks L, Chao KS, Nam J, Eisbruch A. Radiotherapy dose–volume effects on salivary gland function. Int J Radiat Oncol Biol Phys 2010;76:58-63.
14. Li Y, Taylor JM, Ten Haken RK, Eisbruch A. The impact of dose on parotid salivary recovery in head and neck cancer patients treated with radiation therapy. Int J Radiat Oncol Biol Phys 2007;67:660-9.
15. Murdoch-Kinch CA, Kim HM, Vineberg KA, Ship JA, Eisbruch A. Dose-effect relationships for the submandibular salivary glands and implications for their sparing by intensity modulated radiotherapy. Int J Radiat Oncol Biol Phys 2008;72:373–82.
16. Buus S, Grau C, Munk OL, Rodell A, Jensen K, Mouridsen K, et al. Individual radiation response of parotid glands investigated by dynamic 11C-methionine PET. Radiother Oncol 2006;78:262-9.

17. Eisbruch A, Ten Haken RK, Kim HM, Marsh LH, Ship JA. Dose, volume, and function relationships in parotid salivary glands following conformal and intensity-modulated irradiation of head and neck cancer. Int J Radiat Oncol Biol Phys 1999; 45:577-87.
18. Braam PM, Terhaard CH, Roesink JM, Raaijmakers CP. Intensity-modulated radiotherapy significantly reduces xerostomia compared with conventional radiotherapy. Int J Radiat Oncol Biol Phys 2006; 66:975-80.
19. Fiorentino A, Caivano R, Metallo V, Chiumento C, Cozzolino M, Califano G, et al. Parotid gland volumetric changes during intensity-modulated radiotherapy in head and neck cancer. Br J Radiol 2012;85:1415-9.
20. Nagler RM, Baum BJ, Miller G, Fox PG. Long-term salivary effects of single-dose head and neck irradiation in the rat. Arch Oral Biol 1998;43:297-303.
21. Radfar L, Sirois DA. Structural and functional injury in minipig salivary glands following fractionated exposure to 70 Gy of ionizing radiation: an animal model for human radiation-induced salivary gland injury. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;96:267-74.
22. Ricchetti F, Wu B, McNutt T, Wong J, Forastiere A, Marur S, et al. Volumetric change of selected organs at risk during IMRT for oropharyngeal cancer. Int J Radiat Oncol Biol Phys 2011;80:161–8.
23. Hakim SG, Kosmehl H, Lauer I, Nadrowitz R, Wedel T, Sieg P. A comparative study on the protection profile of lidocaine, amifostine, and pilocarpin on the parotid gland during radiotherapy. Cancer Res 2005;65:10486-93.

24. Henriksson R, Fröjd O, Gustafsson H, Johansson S, Yi-Qing C, Franzén L, et al. Increase in mast cells and hyaluronic acid correlates to radiation-induced damage and loss of serous acinar cells in salivary glands: the parotid and submandibular glands differ in radiation sensitivity. Br J Cancer 1994;69:320-6.
25. Stephens LC, King GK, Peters LJ, Ang KK, Schultheiss TE, Jardine JH. Acute and late radiation injury in rhesus monkey parotid glands. Evidence of interphase cell death. Am J Pathol 1986;124:469-78.
26. Xu J, Yan X, Gao R, Mao L, Cotrim A, Zheng C, et al. Effect of irradiation on microvascular endothelial cells of parotid glands in the miniature pig. Int J Radiat Oncol Biol Phys 2010;78:897-903.
27. Coopes RP, Vissink A, Konings AW. Comparison of radiosensitivity of rat parotid and submandibular glands after different radiation schedules. Radiother Oncol 2002;63:321-8.
28. Limesand KH, Avila JL, Victory K, Chang HH, Shin YJ, Grundmann O, et al. Insulin-like growth factor–1 preserves salivary gland function after fractionated radiation. Int J Radiat Oncol Biol Phys 2010;78:579-86.
29. Ziegler U, Groscurth P. Morphological features of cell death. News Physiol Sci 2004;19:124-8.
30. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006;25:4798-811.
31. Prise KM, Schettino G, Folkard M, Held KD. New insights on cell death from radiation exposure. Lancet Oncol 2005;6:520-8.
32. Desagher S, Martinou JC. Mitochondrial as the central control point of apoptosis. Trends Cell Biol 2000;10:369-76.
33. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000;6:513-6.

34. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999;397:441–6.
35. Martin KL, Hill GA, Klein RR, Arnett DG, Burd R, Limesand KH. Prevention of radiation-induced salivary gland dysfunction utilizing a CDK inhibitor in a mouse model. PLoS One 2012;7:e51363.
36. Hai B, Yang Z, Shangguan L, Zhao Y, Boyer A, Liu F. Concurrent transient activation of Wnt/b-catenin pathway prevents radiation damage to salivary glands. Int J Radiat Oncol Biol Phys 2012; 83:e109-16.
37. Chen JL, Lin HH, Kim KJ, Lin A, Forman HJ, Ann DK. Novel roles for protein kinase Cdelta-dependent signaling pathways in acute hypoxic stress-induced autophagy. J Biol Chem 2008; 283:34432–44.
38. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132:27-42.
39. Ahlner BH, Lind MG. The effect of irradiation on blood flow through rabbit submandibular glands. Eur Arch Otorhinolaryngol 1994;251:72-5.
40. Savage NW, Kruger BJ, Adkins KF. Rat submandibular gland microvasculature following fractionated megavoltage irradiation. Aust Dent J 1985;30:99–103.
41. Desmons S, Heger M, Delfosse C, Falgayrac G, Sarrazin T, Delattre C, et al. A preliminary investigation into the effects of x-ray radiation on superficial cranial vascularization. Calcif Tissue Int 2009;84:379-87.
42. Yarnold J, Brotons MC. Pathogenetic mechanisms in radiation fibrosis. Radiother Oncol 2010; 97:149–61.

43. Forrester H, Ivashkevich A, McKay MJ, Leong T, de Kretser DM, Sprung CN. Follistatin is induced by ionizing radiation and potentially predictive of radiosensitivity in radiation- induced fibrosis patient derived fibroblasts. PLoS One 2013; 8:e77119.
44. Friedrich RE, Bartel-Friedrich S, Holzhausen HJ, Lautenschlager C. The effect of external fractionated irradiation on the distribution pattern of extracellular matrix proteins in submandibular salivary glands of the rat. J Craniomaxillofac Surg 2002;30:246–54.
45. Hakim SG, Ribbat J, Berndt A, Richter P, Kosmehl H, Benedek GA, et al. Expression of Wnt-1, TGF- β and related cell-cell adhesion components following radiotherapy in salivary glands of patients with manifested radiogenic xerostomia. Radiother Oncol 2011;101:93-9.
46. Houweling AC, Schakel T, van den Berg CA, Philippens ME, Roesink JM, Terhaard CH, et al. MRI to quantify early radiation-induced changes in the salivary glands. Radiother Oncol 2011;100:386-9.
47. Hakim SG, Benedek GA, Su YX, Jacobsen HC, Klinger M, Dendorfer A, et al. Radioprotective effect of lidocaine on function and ultrastructure of salivary glands receiving fractionated radiation. Int J Radiat Oncol Biol Phys 2012;82:e623-30.
48. Medina VA, Prestifilippo JP, Croci M, Carabajal E, Bergoc RM, Elverdin JC, et al. Histamine prevents functional and morphological alterations of submandibular glands induced by ionising radiation. Int J Radiat Biol 2011;87:284-92.
49. Williamson RA. An experimental study of the use of hyperbaric oxygen to reduce the side effects of radiation treatment for malignant disease. Int J Oral Maxillofac Surg 2007;36:533–40.

50. Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol 2008;9:402–12.
51. Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M. Regulation of autophagy by cytoplasmic p53. Nat Cell Biol 2008;10:676–87.
52. Di Leonardo A, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev 1994;8:2540–51.
53. Grundmann O, Fillinger JL, Victory KR, Burd R, Limesand KH. Restoration of radiation therapy-induced salivary gland dysfunction in mice by post therapy IGF-1 administration. BMC Cancer 2010;10:417.
54. Cohen-Jonathan E, Toulas S, Monteil B, Counderc B, Maret A, Bard J, et al. Radioresistance induced by the high molecular forms of the basic fibroblast growth factor is associated with an increased G2 delay and a hyperphosphorylation of p34CDC2 in HeLa cells. Cancer Res 1997;57:1364–70.
55. Haimovitz-Friedman A, Balaban M, McLoughlin D, Ehleiter J, Michaeli I, Vlodavsky I, et al. Protein kinase C mediates basic fibroblast growth-factor protection of endothelial cells against radiation-induced apoptosis. Cancer Res 1994;54:2591–7.
56. Okazaki Y, Kagami T, Hattori S, Hishida T, Shigetomi, Ueda M. Acceleration of rat salivary gland tissue repair by basic fibroblast growth factor. Arch Oral Biol 2000;45:911–9.
57. Thula TT, Schultz G, Tran-Son-Tay R, Batich C. Effects of EGF and bFGF on irradiated parotid glands. Ann Biomed Eng 2005; 33:685-95.

58. Takeoka M, Ward WF, Pollack H, Kamp DW, Panos RJ. KGF facilitates repair of radiation-induced DNA damage in alveolar epithelial cells. Am J Physiol 1997;272:L1174–80.
59. Buckley S, Barsky L, Driscoll B, Weinberg K, Anderson KD, Warburton D. Apoptosis and DNA damage in type 2 alveolar epithelial cells cultured from hyperoxic rats. Am J Physiol 1998;274:L714 –20.
60. Braun S, Krampert M, Bodo E, Kümin A, Born-Berclaz C, Paus R, et al. Keratinocyte growth factor protects epidermis and hair follicles from cell death induced by UV irradiation, chemotherapeutic or cytotoxic agents. J Cell Sci 2006;119:4841–9.
61. Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PloS One 2008; 3:e2063.
62. Nanduri LS, Lombaert IM, van der Zwaag M, Faber H, Brunsting JF, van Os RP, et al. Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. Radiother Oncol 2013;108:458-63.
63. Banh A, Xiao N, Cao H, Chen CH, Kuo P, Krakow T, et al. A novel aldehyde dehydrogenase-3 activator leads to adult salivary stem cell enrichment in vivo. Clin Cancer Res 2011;17:7265–72.
64. Xiao N, Cao H, Chen CH, Kong CS, Ali R, Chan C, et al. A novel aldehyde dehydrogenase-3 activator (Alda-89) protects submandibular gland function from irradiation without accelerating tumor growth. Clin Cancer Res 2013; 19:4455-64.
65. Huang H, He X. Wnt/beta-catenin signaling: new (and old) players and new insights. Curr Opin Cell Biol 2008;20:119–25.

66. Goessling W, North TE, Loewer S, Lord AM, Lee S, Stoick-Cooper CL, et al. Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. Cell 2009;136:1136–47.
67. Tan X, Behari J, Cieply B, Michalopoulos GK, Monga SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. Gastroenterology 2006;131:1561–72.
68. Jham BC, Teixeira IV, Aboud CG, Carvalho AL, Coelho Mde M, Freire AR. A randomized phase III prospective trial of bethanechol to prevent radiotherapy-induced salivary gland damage in patients with head and neck cancer. Oral Oncol 2007;43:137-42.
69. Peter B, van Waarde MA, Vissink A, s-Gravenmade EJ, Konings AW. The role of secretory granules in radiation-induced dysfunction of rat salivary glands. Radiat Res 1995;141:176–82.
70. Ono K, Inagaki T, Iida T, Wakasugi-Sato N, Hosokawa R, Inenaga K. Distinct effects of cevimeline and pilocarpine on salivary mechanisms, cardiovascular response and thirst sensation in rats. Arch Oral Biol 2012;57:421-8.
71. Xiang B, Zhang Y, Li YM, Gao Y, Gan YH, Wu LL, et al. Phenylephrine protects autotransplanted rabbit submandibular gland from apoptosis. Biochem Biophys Res Commun 2008;377:210-4.
72. Xiang B, Li YJ, Zhao XB, Zou Y, Yu ZG, Zhao YM, et al. Mechanism of the protective effect of phenylephrine pretreatment against irradiation-induced damage in the submandibular gland. Exp Ther Med 2013;5:875-9.
73. Kim JH, Park SH, Moon YW, Hwang S, Kim D, Jo SH, et al. Histamine H1 receptor induces cytosolic calcium increase and aquaporin translocation in human salivary gland cells. J Pharmacol Exp Ther 2009;330:403–12.

74. Medina V, Cricco G, Nuñez M, Martín G, Mohamad N, Correa-Fiz F, et al. Histamine-mediated signaling processes in human malignant mammary cells. Cancer Biol Ther 2006;5:1462–71.
75. Medina VA, Prestifilippo JP, Croci M, Carabajal E, Bergoc RM, Elverdin JC, et al. Histamine prevents functional and morphological alterations of submandibular glands induced by ionising radiation. Int J Radiat Biol 2011;87:284–92.
76. Bertsche U. Induction and repair of X-ray damage in mammalian cell cultures treated with membrane-active drugs. Br J Cancer Suppl 1984;6:121–7.
77. Kang TS, Gorti GK, Quan SY, Ho M, Koch RJ. Effect of hyperbaric oxygen on the growth factor profile of fibroblasts. Arch Facial Plast Surg 2004;6:31–5.
78. Marx RE, Ehler IA, Tayapongsak P, Pierce LW. Relationship of oxygen dose to angiogenesis induction in irradiated tissue. Am J Surg 1990;160:519–24.
79. Thom SR, Bhopale VM, Velasquez OC, Goldstein LJ, Thom LH, Buerk DG. Stem cell mobilization by hyperbaric oxygen. Am J Physiol Heart Circ Physiol 2006;290:1378–86.
80. Thorn JJ, Kallehave F, Westergaard P, Hansen EH, Gottrup F. The effect of hyperbaric oxygen on irradiated oral tissues: transmucosal oxygen tension measurements. J Oral Maxillofac Surg 1997;55:1103–7.
81. Teymoortash A, Sommer F, Mandic R, Schulz S, Bette M, Aumüller G, et al. Intraglandular application of botulinum toxin leads to structural and functional changes in rat acinar cells. Br J Pharmacol 2007;152:161–7.
82. Abok K, Brunk U, Jung B, Ericsson J. Morphologic and histochemical studies on the differing radiosensitivity of ductular and acinar cells of the rat submandibular gland. Virchows Arch B Cell Pathol Incl Mol Pathol 1984;45:443–60.

83. Nagler RM. Effects of head and neck radiotherapy on major salivary glands - animal studies and human implications. In Vivo 2003;17:369–75.

84. Teymoortash A, Müller F, Juricko J, Bieker M, Mandic R, Librizzi D, et al. Botulinum toxin prevents radiotherapy-induced salivary gland damage. Oral Oncol 2009;45:737-9.

4 ARTIGO DE PESQUISA

4 ARTIGO DE PESQUISA

EFFECT OF LOW-LEVEL LASER THERAPY ON IRRADIATED PAROTID GLANDS – A STUDY IN MICE

Artigo submetido para avaliação (Anexo D)

Periódico: Jounal of Biomedical Optics

Qualis Capes Odontologia 2013: A1

Fator de Impacto: 2,75

EFFECT OF LOW-LEVEL LASER THERAPY ON IRRADIATED PAROTID GLANDS – A STUDY IN MICE

Monique Dossena Acauan*

Ana Paula Neutziling Gomes**

Aroldo Braga-Filho***

Maria Antonia Zancanaro de Figueiredo*

Karen Cherubini*

Fernanda Gonçalves Salum*

***Oral Medicine Division, São Lucas Hospital, Pontifical Catholic University of Rio Grande do Sul- PUCRS, Brazil.**

****Oral Pathology Division, Federal University of Pelotas-UFPEL, Brazil.**

*****Radiotherapy Division, São Lucas Hospital, Pontifical Catholic University of Rio Grande do Sul- PUCRS, Brazil.**

Corresponding address:

Fernanda Gonçalves Salum

Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS

Hospital São Lucas

Av. Ipiranga, 6690 – Room 231

CEP: 90610-000 - Porto Alegre – RS – Brazil

Tel/Fax: +55 51 3320-3254

E-mail: fernanda.salum@pucrs.br

ABSTRACT

The objective of this study was to evaluate the effect of low-level laser therapy (LLLT) on morphological alterations and immunodetection of caspase-3 protein in the parotid glands of irradiated mice. Forty-one Swiss mice were divided into a control group and three experimental groups: radiotherapy, 2 J laser and 4 J laser. The experimental groups were exposed to ionizing radiation in a single session of 10 Gy. In the laser groups, a GaAlAs laser (830 nm, 100 mW, 0.028 cm^2, 3.57 W/cm^2) was used on the region corresponding to the parotid glands, with 2 J energy (20 sec, 71 J/cm^2) or 4 J (40 sec, 135 J/cm^2) per point. The animals were euthanized 48 hours or seven days after radiotherapy and parotid glands were dissected for morphological analysis and immunodetection of caspase-3. There was no significant difference between groups in the immunodetection of caspase-3, but the laser group had a lower percentage compared to the radiotherapy group. Furthermore, the results indicated that LLLT promoted the preservation of acinar structure, reduced the occurrence of vacuolation and stimulated parotid gland vascularization. Of the two LLLT protocols, the one using 4 J of energy showed better results.

Keywords: Radiotherapy, Salivary gland, Low Level Laser, Morphology, Apoptosis.

INTRODUCTION

Head and neck radiotherapy often involves the major salivary glands, which undergo morphological and functional changes, resulting in hyposalivation and xerostomia. Approximately 70% of patients irradiated in the head and neck have a progressive loss of salivary gland function (1). Quantitative and qualitative salivary changes cause a total or partial loss of taste, pain and burning mouth, dysphagia, dysphonia, increased susceptibility to oral infections and dental caries, among other complications (2).

There are no studies that clearly show how radiotherapy acts on the function of the salivary glands. Although they are stable because they do not have a high mitotic rate, acinar cells respond quickly to radiation (3-6). The parotid gland, accounting for approximately 60% of saliva, is considered the most radiosensitive of the major salivary glands (4,7-9). The acute and late changes observed in irradiated glands include loss and atrophy of the acinar cells, decrease in glandular weight and formation of fibrous tissue (4,5,10,11).

In an attempt to avoid the adverse effects of radiotherapy in the salivary glands, studies have tested different methods of prevention and treatment of xerostomia. These include especially cytoprotective agents, growth factors, muscarinic cholinergic agonists and low-level laser radiation (4,5,12-17). In general, low-level laser therapy (LLLT) uses light energy in the form of photons to produce cellular responses (18). Its action on tissues can regulate the synthesis of nucleic acids and proteins and modulate the levels of cytokines, growth factors and inflammatory mediators, as well as stimulating cell proliferation and differentiation (19).

Simões et al. (20) used LLLT on the major salivary glands of mice and observed an increase in salivary flow at the end of treatment. Simões et al. (16) evaluated the response of the salivary glands to LLLT in patients undergoing radiotherapy. When applied concomitantly with head and neck radiotherapy, LLLT prevented the reduction in salivary flow. Loncar et al. (21) applied LLLT to the parotid, submandibular and sublingual glands of patients with xerostomia, for ten consecutive days. The authors observed that the amount of saliva produced in the laser group increased gradually during the study. Onizawa et al. (22), in an *in vitro* study with mouse parotid acinar cells, observed that LLLT induced cell proliferation and increased expression of the antiapoptotic proteins Bcl-2 and HSP25.

Considering the evidences before mentioned, the aim of this study was to evaluate the effect of LLLT on radiotherapy-induced morphological changes and immunodetection of caspase-3 protein in parotids of mice.

MATERIALS AND METHODS

The study was approved by the Ethics Committee on Animal Use (CEUA) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS). The sample consisted of 41 male Swiss mice, weighing 25-30 g at the beginning of the experiment. The animals were kept in the Center for Experimental Biological Models of PUCRS in temperature-controlled (23 $\pm$ 1ºC) chambers equipped with input and output air filters, and with a 12 h light-dark cycle. They were housed in cages appropriate for rodents with free access to water and food.

The animals were randomly divided into four groups: control group (n = 5), radiotherapy group (n = 12), 2 Joules (J) laser group (n = 12) and 4 J laser group (n =

12). The radiotherapy group and 2 J and 4 J laser groups were divided into two experimental times, that is, six animals were euthanized 48 hours after radiotherapy and the other animals, seven days after receiving the ionizing radiation. The study flow diagram is shown in Figure 1.

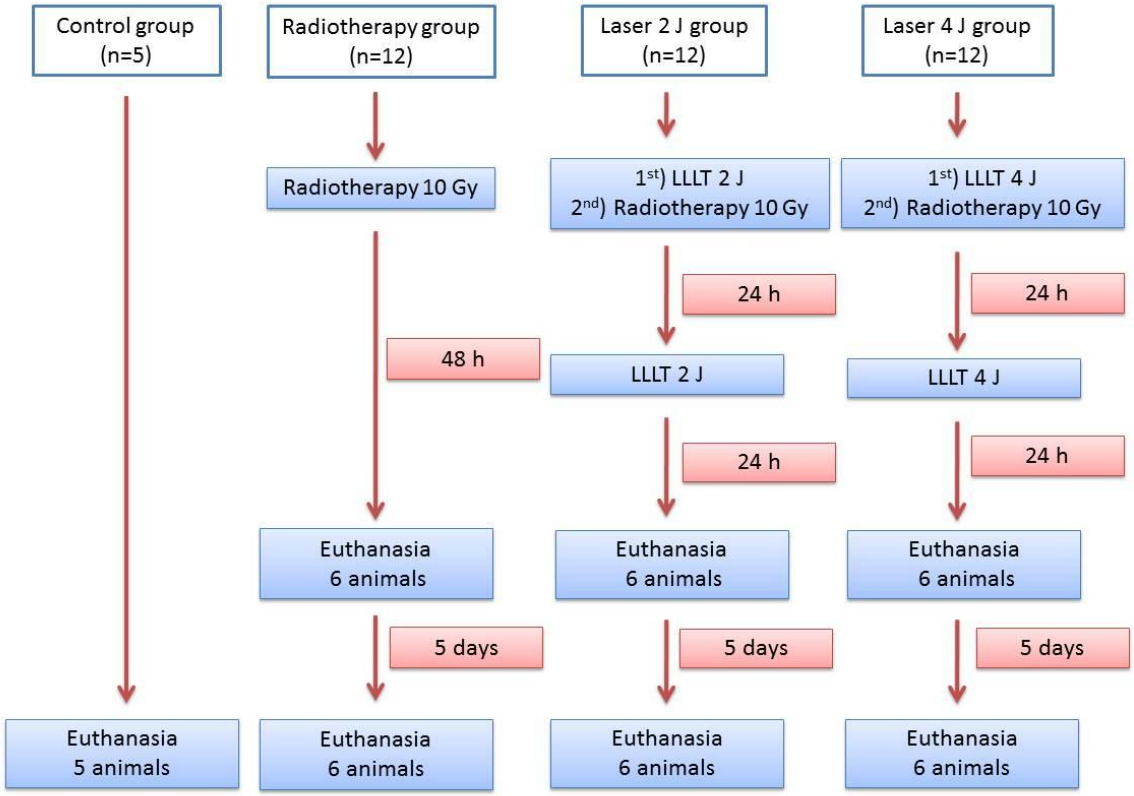

Figure 1. Flowchart representing the stages of the study.

Radiotherapy

Radiotherapy was performed in a single session in the Radiotherapy Department of the São Lucas Hospital. During radiotherapy, the animals were immobilized by means of a restraint for mice up to 50 g (Insight EB 286P, Brazil). The animals were placed in the prone position and irradiated with Co^{60} using a teletherapy unit (Philips, XK5101, Netherlands). The radiation dose used was 10 grays (Gy),

based on the study of Limesand et al. (5). The yield of the radiation source was 60.91 cGy/min, the distance between the emission point of the radioactive beam and the animals was 54.5 cm and the area of the radiation field was 20 cm X 20 cm. The animals of the radiotherapy group and 2 J and 4 J laser groups were subjected to ionizing radiation.

Low-Level Laser Therapy

A GaAlAs diode laser was used (Thera Lase, DMC Equipment Ltda, Brazil); the area of the spot tip of this tool was 0.028 cm^2. Laser irradiation was performed in continuous wave mode. The following parameters were used: 830 nm (infrared) wavelength, 100 mW output power, 3.57 W/cm^2 power density.

- 2 J laser group: we used 71 J/cm^2 dose, 2 J energy, 20 seconds application time.
- 4 J laser group: we used 135 J/cm^2 dose, 4 J energy, 40 seconds application time.

LLLT was performed on the laser groups immediately before and after 24 hours the radiotherapy. The spot tip was placed in contact with the mouse skin in the region corresponding to the parotid glands. One point was applied in each parotid gland. The angle of incidence of the light beam on the tissue was as perpendicular as possible, minimizing refraction. The laser was calibrated before each LLLT session; the laser device had a calibration system coupled to the instrument. Furthermore, after calibration, we used the power meter to check the output power.

Euthanasia and Preparation of Tissues

The animals were sacrificed using a CO_2 chamber. In the radiotherapy and 2 J and 4 J laser groups, six mice were euthanized 48 hours after radiotherapy and the other animals after seven days. At this time, the animals in the control group were also euthanized.

The right and left parotid glands of each animal were dissected and immersed for 24 h in 10% buffered formalin. They were then dehydrated in increasing concentrations of alcohol, cleared and embedded in paraffin. The left and right glands of each animal were included in the same paraffin block, from which two 3-µm thick sections were obtained. One section was stained with hematoxylin-eosin (HE) and the other processed to immunohistochemical with anti-caspase-3 antibody.

For immunohistochemistry, the section was mounted on a silanized slide (Flex DAKO, USA). These slides were subjected to immunohistochemistry with anti-cleaved caspase-3 antibody (1:300) (Cell Signaling # 9661, USA). Accordingly, histological sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series. Antigen retrieval was performed using citric acid in a water bath at 95°C for 30 min. Endogenous peroxidase block was performed with hydrogen peroxide diluted 20 times with methanol. Incubation with primary antibody was for 60 min at room temperature. The detection system used was EasyLink One (EasyPath, Brazil) and the development of the reaction was with the DAB chromogen kit (EasyPath, Brazil). Slides were counter-stained with Mayer's hematoxylin and dehydrated in an increasing ethanol series. The sections were cleared with xylol, and glass coverslips were mounted with Permount (Fisher Scientific, USA). Normal lymph node served as the positive control for the reactions. Negative controls were obtained by omitting the primary antibody.

Histological evaluation of the glandular tissue was performed by a single blinded and calibrated examiner. Initially, we carried out a descriptive analysis of the HE-stained slides, observing the acinar structure (acinar disorganization), vascularization, presence of cytoplasmic vacuolation, inflammatory infiltrate, fibrosis and edema. For each parameter the following histological scores were established: absent (-); slight (+); moderate (++) or severe (+++). Those parameters not observed on the slides were considered absent. The score was considered slight when the histological parameter was observed in isolated areas on the slide. A severe score was given when the parameter was observed distributed in the entire slide. When an intermediate pattern was found, between slight and severe changes, the score was considered moderate. A slight score (+) was given for vascularization within the normal range as observed in non-irradiated glands, while a moderate score was considered when the amount of blood vessels was greater than normal.

For immunodetection of caspase-3, we selected from each slide five equidistant fields, captured at 200X magnification using an image capture system (Moticam 5 - System ShiftCapture, China) connected to a light microscope (Olympus BX50, Japan). Captured images were saved in TIFF format and analyzed using the software ImageJ 1.48v. In each image, an automated analysis was performed to quantify the area staining positive for caspase-3 in acinar cells. Immunostaining percentage was determined for the five fields analyzed. Areas corresponding to ducts and blood vessels were omitted to avoid an error in detection.

Data Analysis

The data were analyzed using descriptive statistics. We used the Kruskal-Wallis test to compare the percentage of caspase-3 immunostaining between groups.

To compare the different time points within each group, the Mann-Whitney test was used at a significance level of 5%. Statistical analysis was performed with SPSS version 18.0.

RESULTS

Morphological Analysis

The control group showed normal acinar structure and vascularization (Figure 2A and 2B), but some vacuolated cells were seen. The glands of the animals in the radiotherapy group showed marked acinar disorganization, characterized by altered morphology and size of the acini, which was most significant 48 hours after radiation. Also in this group, the presence of vacuolated cells was more evident than in the control, mainly seven days after radiotherapy.

In the 2 J and 4 J laser groups, 48 hours after radiotherapy, cytoplasmic vacuolation was similar to that observed in the control group. Acinar disorganization areas were present but were less evident than in the radiotherapy group. Greater vascularization was observed in the glands of the 4 J laser group at this experimental time.

Seven days after radiotherapy, the 2 J and 4 J laser groups had higher vascularization compared to the other groups. At this time, the presence of vacuolated cells was more pronounced compared to the control group, but less evident than in the radiotherapy group. As for acinar structure, the pattern in the 4 J laser group at seven days after radiation was the same as that observed at 48 hours. In the 2 J laser group, we observed marked acinar disorganization, resembling that in

the radiotherapy group (Figure 2D). There were no areas of fibrosis, inflammatory infiltrate or edema in the study groups.

Table 1. Changes in glandular morphology based on descriptive analysis, in the control, 2 J laser, 4 J laser and radiotherapy groups, at different time points (48 hours and 7 days).

GROUPS		Acinar Disorganization	Vacuolation	Vascularization
Control		-	+	+
2 J Laser	48h	+	+	+
	7 days	++	++	++
4 J Laser	48h	+	+	++
	7 days	+	++	++
Radiotherapy	48h	+++	++	+
	7 days	++	+++	+

(-) absent; (+) slight; (++) moderate; (+++) severe.

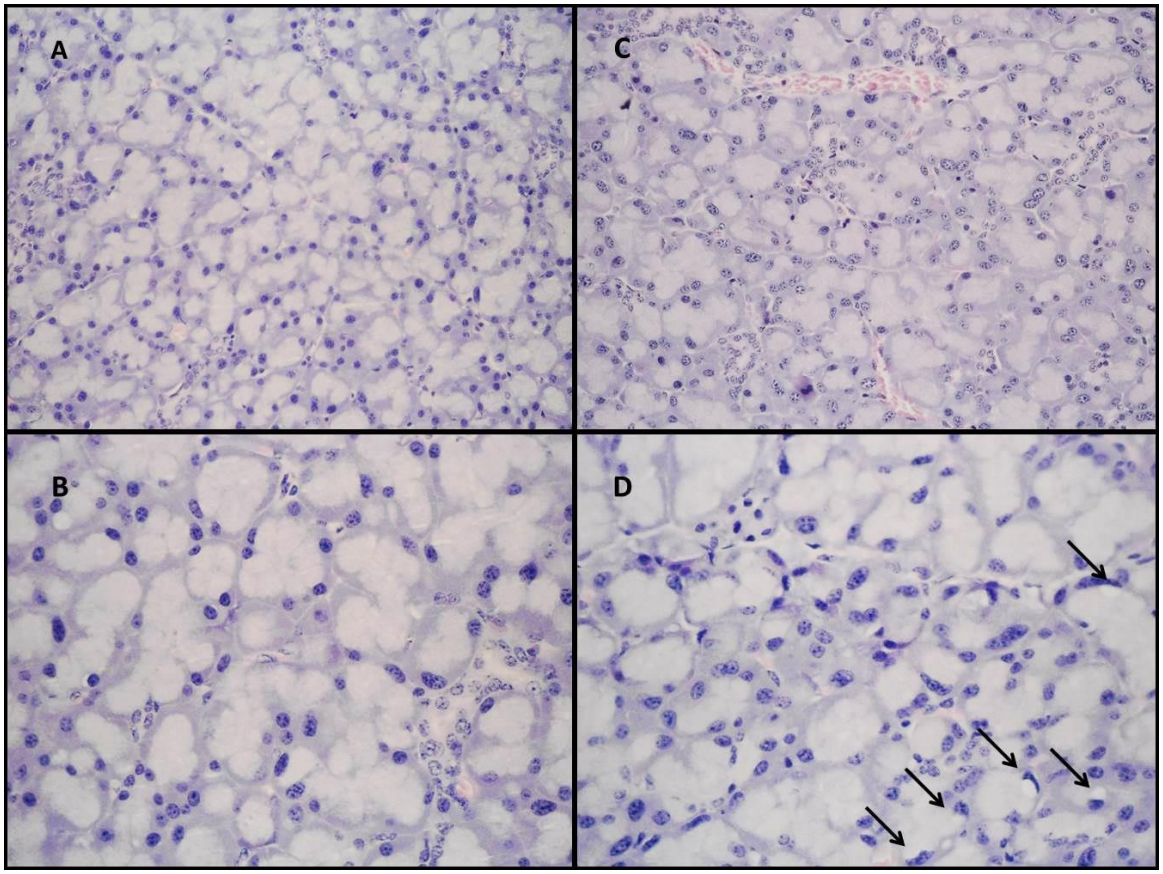

Figure 2. Histologic examination of parotid gland structure. Control group, X200 (A) and X400 (B) showing normal acinar structure. 4 J laser group, 48 h after radiotherapy, showing greater vascularization (C). 2 J laser group, seven days after radiotherapy, displaying acinar disorganization and vacuolated cells (arrow) (D).

Immunodetection of caspase-3

There was no significant difference in caspase-3 immunodetection between groups or between time points (Table 2). However, the data showed a lower percentage in the control group and greater percentage in the radiotherapy group. In the laser groups, percentage of caspase-3 immunodetection was intermediate between the values found in the radiotherapy and control groups.

Table 2. Percentage of caspase-3 immunodetection in the radiotherapy, 2 J laser, 4 J laser and control groups, at different time points (48 hours and 7 days).

	48 hours	**7 days**	
GROUPS	Median (P25-P75)	Median (P25-P75)	*P***
Radiotherapy	0.64 (0.19-1.41)	0.53 (0.3-1.02)	*1.0*
2 J Laser	0.17 (0.02-0.63)	0.32 (0.09-0.75)	*0.42*
4 J Laser	0.15 (0.07-1.29)	0.26 (0.02-1.46)	*1.0*
Control	0.05 (0.03-0.1)	0.05 (0.03-0.1)	-
*P**	*0.06*	*0.085*	

* Kruskal-wallis and ** Mann-whitney tests significant at p≤0.05.

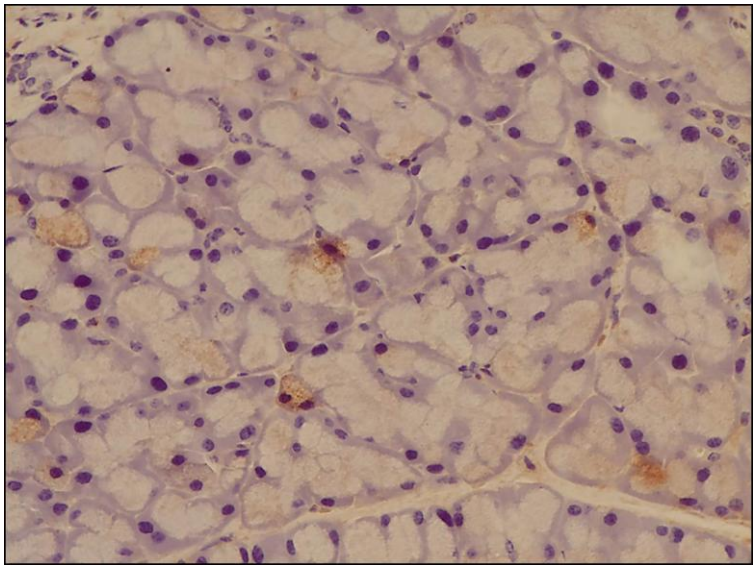

Figure 3. Caspase-3 immunostaining in parotid gland in 4 J laser group, seven days after radiotherapy.

DISCUSSION

The dysfunction of the salivary glands is a frequent complication of head and neck radiotherapy and is directly related to structural damage (23). Changes in acinar structure are described within the first 48 and 72 hours after radiotherapy (24). The acute effects of ionizing radiation on the salivary glands appear to be due to high levels of cell death, and many studies have suggested that chronic effects may be due to the damage initially produced (9,24). The present study investigated the effect of LLLT on acute morphological changes and on the detection of caspase-3 protein in parotid glands of irradiated mice.

In this study, in both laser groups, 48 hours after radiotherapy, cytoplasmic vacuolation as well as acinar structure appeared similar to that found in the control group. These results indicate that LLLT could have preserved glandular morphology during the first hours after exposure to ionizing radiation. Seven days after radiotherapy, however, there was an increase in cytoplasmic vacuolation and changes in acinar structure, which were more pronounced in the 2 J laser group. However, most of these changes were still less evident than that observed in the radiotherapy group. In our study, LLLT was applied in two sessions, i.e., immediately before and 24 h after radiation. If more sessions of LLLT had been used in this seven-day period, it is possible that the morphological changes found a week after radiotherapy could have been less evident.

Several authors have described the occurrence of cytoplasmic vacuolation in salivary glands of irradiated animals (6,24-26), evident in the first hours after radiotherapy (6,24,25).The vacuoles in the cytoplasm represent an active process of autophagy (27), induced by nutritional shortage, infection or oxidative stress (28).

Vacuolated cells were more evident in the radiotherapy group than the other groups. However, even the salivary glands in the control group showed some vacuolated cells. The presence of cytoplasmic vacuolation was also recorded by Radfar and Sirois (11) in salivary glands of non-irradiated minipigs.

The salivary glands of the laser group had higher vascularization compared to control and radiotherapy groups. Laser stimulation of microcirculation has been described by other authors, when applying LLLT to different tissues (29,30). On the other hand, the literature demonstrates that salivary glands of irradiated animals display hypovascularization with changes in blood flow and distribution of vessels immediately after radiotherapy (26,31,32). These alterations were not observed in this study when comparing the radiotherapy and control groups, a result that can be explained by the difference in the methods for analyzing vascularization, since in the literature, other techniques such as immunohistochemistry and doppler have been used (26,31,32).

In this study, there were no areas of fibrosis, inflammatory infiltrate or edema. These results are consistent with most studies reporting the occurrence of fibrosis as a late effect in irradiated salivary glands, where it is found more often after 30 days post-irradiation (6,11,33). The presence of inflammatory infiltrate in salivary glands resulting from radiotherapy is rarely mentioned in the literature, and only observed in studies using minipigs and rhesus monkeys as experimental model (24,34). The occurrence of edema, although not manifesting late, was quoted in the literature only on the tenth day after exposure to ionizing radiation, which would explain why we did not observe this up to the seventh day (6).

Since apoptosis of acinar cells has been identified as an acute event caused by radiotherapy (4,5,27,35), which influences the loss of function of the salivary

glands, we chose to investigate the immunodetection of caspase-3 protein. Although not significantly different between the groups, the percentage of caspase-3 immunostaining was higher in the radiotherapy group and lower in the control group, while the laser groups showed intermediate values, indicating that LLLT might have influenced acinar cell apoptosis.

In the literature, quantification of caspase-3 in the salivary glands of irradiated mice varied 2 to 27%, depending on the radiation dose and observation time (4,5,27,36). These percentages are higher than those found in our study, probably because of the quantification methods used. In these studies, the percentage was obtained by the number of labeled cells. In our study, caspase-3 was quantified by the stained area in relation to the total area of each histological field, because cytoplasmic staining appeared diffuse in some slides and because cell counts alone could produce bias. Another issue to be considered is that studies differ on the peak occurrence of apoptosis. Avila et al. (4) observed in transgenic animals an increased number of apoptotic cells 48 hours after radiation. In the study by Limesand et al. (36), the expression of caspase-3 was higher 24 hours after radiotherapy, decreasing drastically when assessed at 48 hours. The authors explained this result as being due to the removal of these cells by phagocytosis (27), which could also explain the low percentage of detection of this protein in our study.

The lack of a therapeutic protocol for LLLT made methodological definitions in this study difficult. In the literature, laser parameters differ considerably in studies using LLLT to treat xerostomia (16,21). There have been differences in wavelength, power, energy and frequency of sessions (7,16,21). We opted for an infrared wavelength, due to the depth of glandular parenchyma to be irradiated (21). The determination of the energy of 2 J supplied per point was based on the study of

Saleh et al. (37), and for comparison, we decided to use also an LLLT protocol with energy of 4 J.

The results suggest that when using the protocols defined in this study, LLLT had a tendency to reduce cell apoptosis by reducing active caspase-3. In addition, we suggest that LLLT promoted the preservation of acinar structure, with regard to the organization of acini, reduced the occurrence of vacuolation, and even stimulated the vascularization of the parotid glands of irradiated mice. Of the two LLLT protocols studied, the one using 4 J of energy showed better results. Given the methodological limitations of this study, further researches should be conducted in irradiated animals, using different LLLT protocols and observing glandular response, not only in the short term but also long term, when the occurrence of late changes in the salivary glands can be analyzed.

REFERENCES

1. F.R. Burlage, R.P. Coppes, H. Meertens, et al. “Parotid and submandibular/sublingual salivary flow during high dose radiotherapy”. *Radiother Oncol.* **61**(3):271-4 (2001).
2. P. Dirix, S. Nuyts, V.V. Poorten, et al. “The influence of xerostomia after radiotherapy on quality of life: results of a questionnaire in head and neck cancer”. *Support Care Cancer*. **16**(2):171-9 (2008).
3. G.M. Paardekoope, S. Cammelli, L.J.W. Zeilstra, et al. “Radiation-induced apoptosis in relation to acute impairment of rat salivary gland function”. *Int J Radiat Biol.* **73**(6):641-8 (1998).

4. J.L. Avila, O. Grundmann, R. Burd, et al. “Radiation-induced salivary gland dysfunction results from p53-dependent apoptosis”. *Int J Radiat Oncol Biol Phys*. **73**(2):523–9 (2009).
5. K.H. Limesand, K.L. Schwertfeger, S.M. Anderson. “MDM2 is required for suppression of apoptosis by activated Akt1 in salivary acinar cells”. *Mol Cell Biol*. **26**(23):8840–56 (2006).
6. R.P. Coppes, L.J.W. Zeilstra, H.H. Kampinga, et al. “Early to late sparing of radiation damage to the parotid gland by adrenergic and muscarinic receptor agonists”. *B J Cancer*. **85**(7):1055-63 (2001).
7. A. Simões, S.W. Siqueir, M.L. Lamers, et al. “Laser phototherapy effect on protein metabolism parameters of rat salivary glands”. *Lasers Med Sci*. **24**(2):202–8 (2009).
8. C.A. Murdoch-Kinch, H.M. Kim, K.A. Vineberg, et al. “Dose-effect relationships for the submandibular salivary glands and implications for their sparing by intensity modulated radiotherapy”. *Int J Radiat Oncol Biol Phys*. **72**(2):373–82 (2008).
9. Y. Li, J.M.G. Taylor, R.K.T. Haken, et al. “The impact of dose on parotid salivary recovery in head and neck cancer patients treated with radiation therapy”*. Int J Radiat Oncol Biol Phys.* **67**(3):660-9 (2007).
10. R.M. Nagler, B.J. Baum, G. Miller, et al. “Long-term salivary effects of single-dose head and neck irradiation in the rat”. *Arch Oral Biol*. **43**(4): 297-303 (1998).
11. L. Radfar and D.A. Sirois. “Structural and functional injury in minipig salivary glands following fractionated exposure to 70 Gy of ionizing radiation: an animal model for human radiation-induced salivary gland injury”. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **96**(3):267-74 (2003).

12. B.C. Jham, I.V. Teixeira, C.G. Aboud, et al. “A randomized phase III prospective trial of bethanechol to prevent radiotherapy-induced salivary gland damage in patients with head and neck cancer”. *Oral Oncol.* **43**(2):137-42 (2007).
13. K. Ono, T. Inagaki, T. Lida, et al. “Distinct effects of cevimeline and pilocarpine on salivary mechanisms, cardiovascular response and thirst sensation in rats”. *Arch Oral Biol.* **57**(4):421-8 (2012).
14. D.M. Brizel, T.H. Wasserman, M. Henke, et al. “Phase III randomized trial of amifostine as a radioprotector in head and neck cancer”. *J Clin Oncol.* **18**(19):3339-45 (2000).
15. I.M.A. Lombaert, J.F. Brunsting, P.K. Wierenga, et al. “Rescue of salivary gland function after stem cell transplantation in irradiated glands”. *PloS one.* **3**(4):e2063 (2008).
16. A. Simões, L. Campos, D.N. de Souza, et al. “Laser phototherapy as topical prophylaxis against radiation-induced xerostomia”. *Photomed Laser Surg.* **28**(3):357-63 (2010).
17. O. Grundmann, G.C. Mitchell, K.H. Limesand. “Sensitive of salivary glands to radiation: from animal models to therapies”. *J Dent Res*. **88**(10):894-903 (2009).
18. T. Karu. “Primary and secondary mechanisms of action of visible to near-IR radiation on cells”. *J Photochem Photobiol.* **49**(1):1–17 (1999).
19. J.B. Dawson, D.J Barker, D.J. Ellis, et al. “A theoretical and experimental study of light absorption and scattering by in vivo skin”. *Phys Med Biol.* **25**(4):695–709 (1980).
20. A. Simões, J. Nicolau, D.N. de Souza, et al. “Effect of defocused infrared diode laser on salivary flow rate and some salivary parameters of rats”*. Clin Oral Invest.* **12**(1): 25-30 (2008).

21. B. Loncar, M.M. Stipetic, M. Baricevic, et al. “The effect of low-level laser therapy on salivary glands in patients with xerostomia”. *Photomed Laser Surg*. **29**(3):171–5 (2011).
22. K. Onizawa, T. Muramatsu, M. Matsuki, et al. “Low-level (gallium-aluminum-arsenide) laser irradiation of Par-C10 cells and acinar cells of rat parotid gland”. *Lasers Med Sci*. **24**(2):155-61 (2009).
23. K. Mossman, A. Shatzman, J. Chencharick. “Long-term effects of radiotherapy on taste and salivary function in man”. *Int J Radiat Oncol Biol Phys.* **8**(6):991-7 (1982).
24. L.C. Stephens, G.K. King, L.J. Peters, et al. “Acute and late radiation injury in rhesus monkey parotid glands. Evidence of interphase cell death”. *Am J Pathol.* **124**(3):469-78 (1986).
25. S.G. Hakim, H. Kosmehl, I. Lauer, et al. “A comparative study on the protection profile of lidocaine, amifostine, and pilocarpin on the parotid gland during radiotherapy”. *Cancer Res.* **65**(22):10486-93 (2005).
26. J. Xu, X. Yan, R. Gao, et al. “Effect of irradiation on microvascular endothelial cells of parotid glands in the miniature pig”. *Int J Radiat Oncol Biol Phys.* **78**(3):897-903 (2010).
27. K.H. Limesand, J.L. Avila, K. Victory, et al. “Insulin-like growth factor–1 preserves salivary gland function after fractionated radiation”. *Int J Radiat Oncol Biol Phys.* **78**(2):579-86 (2010).
28. B. Levine and G. Kroemer. “Autophagy in the pathogenesis of disease”. *Cell.* **132**(1):27-42 (2008).

29. A. Domínguez, R.E. Ballesteros, J.H. Viáfara, et al. “Effect of low level laser therapy on dental pulp during orthodontic movement”. *World J Methodol.* **3**(2):19-26 (2013).
30. Y. Maegawa, T. Itoh, T. Hosokawa, et al. “Effects of near-infrared low-level laser irradiation on micro-circulation”. *Lasers Surg Med.* **27**(5):427–37 (2000).
31. B.H. Ahlner and M.G. Lind. “The effect of irradiation on blood flow through rabbit submandibular glands”. *Eur Arch Otorhinolaryngol.* **251**(2):72-5(1994).
32. N.W. Savage, B.J. Kruger, K.F. Adkins. “Rat submandibular gland microvasculature following fractionated megavoltage irradiation”. *Aust Dent J.* **30**(2):99–103 (1985).
33. R. Henriksson, O. Fröjd, H. Gustafsson, et al. “Increase in mast cells and hyaluronic acid correlates to radiation-induced damage and loss of serous acinar cells in salivary glands: the parotid and submandibular glands differ in radiation sensitivity”. *Br J Cancer*. **69**(2):320-6 (1994).
34. J. Li, Z. Shan, G. Ou, et al. “Structural and functional characteristics of irradiation damage to parotid glands in the miniature pig”. *Int J Radiat Oncol Biol Phys.* **62**(5):1510-6 (2005).
35. K.L Martin, G.A. Hill, R.R. Klein, et al. “Prevention of radiation-induced salivary gland dysfunction utilizing a CDK inhibitor in a mouse model”. *PLoS One.* **7**(12):e51363 (2012).
36. K.H. Limesand, S. Said, S.M Anderson. “Suppression of radiation-induced salivary gland dysfunction by IGF-1”. *PLoS One*. **4**(3):e4663 (2009).
37. J. Saleh, M.A. Figueiredo, K. Cherubini, et al. “Effect of low level laser therapy on radiotherapy-induced hyposalivation and xerostomia: a pilot study”. *Photomed Laser Surg*. **32**(10):546-52 (2014).

5 DISCUSSÃO COMPLEMENTAR

5 DISCUSSÃO COMPLEMENTAR

A xerostomia é uma das complicações mais frequentes decorrentes da radioterapia em pacientes com câncer de cabeça e pescoço (9,37). As glândulas salivares são extremamente sensíveis à radiação, mas ao contrário de tecidos classicamente radiossensíveis, apresentam *turnover* lento e são compostas por células altamente diferenciadas (24). Os efeitos prejudiciais da radiação sobre as glândulas salivares são observados logo após o início do tratamento e os indivíduos afetados exibem perda de 50-60% do fluxo salivar na primeira semana após a radioterapia (37-39). A disfunção das glândulas salivares está diretamente relacionada com o dano estrutural (6). Os efeitos agudos da radiação nas glândulas salivares parecem ocorrer devido a altos níveis de morte celular e os efeitos tardios são causados por dano vascular e perda de células parenquimais (16,40).

A radiação laser de baixa potência é uma forma não ionizante e não invasiva de radiação, bem tolerada pelos tecidos e sem efeitos mutagênicos (41). Embora estudos pré-clínicos e clínicos tenham demonstrado os benefícios da TLBP na prevenção e tratamento da xerostomia e hipossalivação (23,35,36,42), seu mecanismo de ação sobre as glândulas salivares não é completamente compreendido. Onizawa et al. (43), em um estudo *in vitro*, demonstraram que a TLBP pode promover a proliferação de células acinares e aumentar a expressão de proteínas anti-apoptóticas. O efeito desta terapia em aumentar a microcirculação sanguínea tecidual também foi demonstrado (44,45). Considerando os dados anteriormente mencionados, o presente estudo investigou o efeito da TLBP sobre alterações morfológicas agudas causadas pela radioterapia em glândulas parótidas de camundongos. A estrutura acinar (desorganização acinar), a vascularização, a

presença de vacuolização citoplasmática, infiltrado inflamatório e edema foram descritas. A presença de fibrose foi também investigada, embora esta seja uma alteração tardia decorrente da radioterapia (13,18,46).

Os resultados do estudo indicam que a TLBP possa ter preservado a morfologia glandular nas primeiras 48 horas após a exposição à radiação ionizante. Além disso, foi observado que a TLBP aumentou a vascularização nas glândulas salivares irradiadas, conforme descrito por outros autores (44,45). Sete dias após a radioterapia, entretanto, houve aumento na vacuolização citoplasmática e alterações na estrutura acinar, o que foi mais acentuado no grupo laser 2 J. A maior parte destas alterações foi ainda menos evidente do que as observadas no grupo radioterapia. Pode-se sugerir que se a TLBP tivesse sido mantida no decorrer do estudo, algumas das alterações morfológicas observadas uma semana após a radioterapia tivessem sido prevenidas nos grupos laser.

No grupo-controle foram observadas algumas células vacuoladas, como também foi registrado por Radfar e Sirois (18) em glândulas salivares de miniporcos. Em animais irradiados, entretanto, a vacuolização citoplasmática é relatada de forma expressiva (13,40,47,48), podendo ser percebida já nas primeiras horas após a radioterapia (13,40,47). Neste estudo, no grupo radioterapia, a presença de células vacuoladas foi mais evidente em comparação aos demais grupos. Os vacúolos no citoplasma representam um processo ativo de autofagia (48), induzido sob escassez nutricional e estresse oxidativo (49).

Não foram encontradas áreas de infiltrado inflamatório e edema neste estudo. A presença de infiltrado inflamatório decorrente da radioterapia em glândulas salivares é pouco citada na literatura, e observada apenas em estudos que utilizaram miniporcos e macacos rhesus como modelo experimental (40,50). A

ocorrência de edema, apesar de não se manifestar tardiamente, foi citada na literatura apenas após o décimo dia de exposição à radiação ionizante, o que explicaria não ter sido observada até o sétimo dia deste estudo (13). Conforme esperado, a presença de fibrose não foi observada no presente estudo.

A apoptose celular é um processo fortemente regulado, que está envolvido na manutenção da homeostase tecidual (51). A ativação da proteína caspase-3 é um evento agudo da apoptose e sua imunodetecção têm sido utilizada por diversos autores para investigar os níveis de morte celular em glândulas salivares irradiadas. A ativação da caspase-3 ocorre em função de a radiação ionizante gerar alterações na permeabilidade da membrana mitocondrial e liberação do citocromo C para o citoplasma. Como consequência, ocorre a liberação da caspase-9, a qual ativa a caspase-3 (52). Há também a perda da homeostase celular, a produção de espécies reativas de oxigénio (ROS) e a interrupção da síntese de ATP. Altos níveis de ROS aumentam ainda mais a permeabilidade da membrana mitocondrial e a ativação das caspases-9 e -3 (53).

O papel da apoptose na disfunção glandular induzida pela radiação tem sido bastante discutido. Avila et al. (11) e Limesand et al. (12) mostraram que a redução da apoptose está diretamente relacionada com o aumento do fluxo salivar de camundongos. A relação entre a proporção de células acinares perdidas após a radiação e a intensidade da disfunção das glândulas salivares têm sido discutida (12,54). Há pelo menos duas correntes: a primeira parte do pressuposto de que todas as células acinares perdidas contribuem igualmente para a diminuição da produção de saliva; a segunda corrente defende a hipótese de que as células apoptóticas afetariam a função de outras células adjacentes.

Uma vez que a apoptose das células acinares tem sido apontada como um evento agudo causado pela radioterapia (11,12,48,55), optamos por investigar a imunodetecção da proteína caspase-3 ativa nas células acinares. Embora não tenha sido observada diferença significativa entre os grupos, o percentual de imunodetecção da caspase-3 foi superior no grupo radioterapia e inferior no grupo-controle, enquanto os grupos laser apresentaram valores intermediários, podendo-se sugerir que a TLBP tenha exercido influência sobre a apoptose das células acinares.

Numerosos estudos têm analisado os danos nas glândulas salivares após a irradiação em diferentes espécies animais incluindo ratos, camundongos, macacos e miniporcos (11-13,18,40,56,57). A principal diferença entre os modelos envolve a dose de radiação administrada necessária para que se possa observar uma perda significativa da função glandular. Em camundongos, doses de radiação entre 1-15 Gy têm sido testadas e, conforme revisado por Grundmann et al. (24), a apoptose celular têm sido observada em doses que variam de 1 a 10 Gy. Portanto, no presente estudo optou-se por utilizar, neste modelo animal, a dose de 10 Gy de radiação e investigar as alterações nas glândulas parótidas, que costumam ser descritas como as mais radiossensitivas das glândulas salivares maiores (15,16).

De acordo com os resultados obtidos, podemos sugerir que, quando aplicada nos protocolos definidos neste estudo, a TLBP apresentou uma tendência em reduzir a apoptose celular, pela redução da atividade da proteína caspase-3. Além disso, podemos sugerir que a TLBP promoveu preservação da estrutura acinar, quanto à organização dos ácinos, reduziu a ocorrência de vacuolização citoplasmática, e ainda estimulou a vascularização das glândulas salivares parótidas de camundongos submetidos à radioterapia. Entre os protocolos de TLBP, a utilização de energia de 4 J, apresentou melhores resultados. Tendo em vista as

limitações metodológicas desta pesquisa, mais estudos devem ser conduzidos em animais irradiados, utilizando diferentes protocolos de TLBP e observando a resposta glandular, não apenas em curto prazo, como também em longo prazo, quando a ocorrência de alterações tardias nas glândulas salivares pode ser analisada.

6 REFERÊNCIAS COMPLEMENTARES

6 REFERÊNCIAS COMPLEMENTARES

1. Segreto HRC, Segreto RA. Revisão e atualização em radiobiologia. Aspectos celulares, moleculares e clínicos. Folha Med 2000; 119:9-27.
2. Pignon JP, Bourhis J, Domenge C, Designé L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. Lancet 2000; 355:949-55.
3. Fu KK, Pajak TF, Trotti A, Jones CU, Spencer SA, Phillips TL, et al. A Radiation Therapy Oncology Group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation for head and neck squamous cell carcinomas. First report of RTOG 9003. Int J Radiat Oncol Biol Phys 2000; 48:7-16.
4. Levendag P, Nijdam W, Noever I, Schmitz P, van de Pol M, Sipkema D, et al. Brachytherapy versus surgery in carcinoma of tonsillar fossa and/or soft palate: late adverse sequelae and performance status: can we be more selective and obtain better tissue sparing? Int J Radiat Oncol Biol Phys 2004; 59:713-24.
5. Lettmaier S, Kreppner S, Lotter M, Walser M, Ott OJ, Fietkau R, et al. Radiation exposure of the heart, lung and skin by radiation therapy for breast cancer: a dosimetric comparison between partial breast irradiation using multicatheter brachytherapy and whole breast teletherapy. Radiother Oncol 2011; 100:189-94.
6. Mossman K, Shatzman A, Chencharick J. Long- term effects of radiotherapy on taste and salivary function in man. Int J Radiat Oncol Biol Phys 1982; 8:991-7.
7. Bhide SA, Ahmed M, Newbold K, Harrington KJ, Nutting CM. The role of intensity modulated radiotherapy in advanced oral cavity carcinoma. J Cancer Res Ther 2012; 8:67-71.

8. Epstein JB, Thariat J, Bensadoun RJ, Barasch A, Murphy BA, Kolnick L, et al. Oral complications of cancer and cancer therapy. CA Cancer J Clin 2012; 62:400–22.
9. Dirix P, Nuyts S, Poorten VV, Delaere P, Van den Bogaert W. The influence of xerostomia after radiotherapy on quality of life. Support Care Cancer 2008; 16:171-9.
10. Paardekooper GMRM, Cammelli S, Zeilstra LJW, Coppes RP, Konings AWT. Radiation-induced apoptosis in relation to acute impairment of rat salivary gland function. Int J Radiat Biol 1998; 73:641-8.
11. Avila JL, Grundmann O, Burd R, Limesand KH. Radiation-induced salivary gland dysfunction results from p53-dependent apoptosis. Int J Radiat Oncol Biol Phys 2009; 73:523–9.
12. Limesand KH, Schwertfeger KL, Anderson SM. MDM2 is required for suppression of apoptosis by activated Akt1 in salivary acinar cells. Mol Cell Biol 2006; 26:8840–56.
13. Coppes RP, Zeilstra LJW, Kampinga HH, Konings AWT. Early to late sparing of radiation damage to the parotid gland by adrenergic and muscarinic receptor agonists. B J Cancer 2001; 85:1055-63.
14. Simões A, Siqueira SW, Lamers ML, Santos MF, Eduardo CP, Nicolau J. Laser phototherapy effect on protein metabolism parameters of rat salivary glands. Lasers Med Sci 2009; 24:202–8.
15. Murdoch-Kinch CA, Kim HM, Vineberg KA, Ship JA, Eisbruch A. Dose-effect relationships for the submandibular salivary glands and implications for their sparing by intensity modulated radiotherapy. Int J Radiat Oncol Biol Phys 2008; 72: 373–82.

16. Li Y, Taylor JMG, Haken RKT, Eisbruch A. The impact of dose on parotid salivary recovery in head and neck cancer patients treated with radiation therapy. Int J Radiat Oncol Biol Phys 2007; 67:660-9.
17. Nagler RM, Baum BJ, Miller G, Fox PG. Long-term salivary effects of single-dose head and neck irradiation in the rat. Arch Oral Biol 1998; 43: 297-303.
18. Radfar L, Sirois DA. Structural and functional injury in minipig salivary glands following fractionated exposure to 70 Gy of ionizing radiation: an animal model for human radiation-induced salivary gland injury. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003; 96:267-74.
19. Jham BC, Teixeira IV, Aboud CG, Carvalho AL, Coelho MM, Freire ARS. A randomized phase III prospective trial of bethanechol to prevent radiotherapy-induced salivary gland damage in patients with head and neck cancer. Oral Oncol 2007; 43:137-42.
20. Ono K, Inagaki T, Lida T, Wakasugi-Sato N, Hosokawa R, Inenaga K. Distinct effects of cevimeline and pilocarpine on salivary mechanisms, cardiovascular response and thirst sensation in rats. Arch Oral Biol 2012; 57:421-8.
21. Brizel DM, Wasserman TH, Henke M, Strnad V, Rudat V, Monnier A et al. Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. J Clin Oncol 2000; 18:3339-45.
22. Lombaert IMA, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PloS One 2008; 3:1-13.
23. Simões A, Campos L, de Souza DN, de Matos JS, Freitas PM, Nicolau. Laser phototherapy as topical prophylaxis against radiation-induced xerostomia. Photomed Laser Surg 2010; 28:357-63.

24. Grundmann O, Mitchell GC, Limesand KH. Sensitive of salivary glands to radiation: from animal models to therapies. J Dent Res 2009; 88: 894-903.
25. Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol 1999; 49:1–17.
26. Sutherland J.C. Biological effects of polychromatic light. Photochem Photobiol 2002; 76:164–70.
27. Karu T. Laser biostimulation: a photobiological phenomenon. J Photochem Photobiol 1989; 3:638–40.
28. Karu TI, Pyatibrat LV, Afanasyeva NI. Cellular effects of low power laser therapy can be mediated by nitric oxide. Lasers Surg Med 2005; 36:307–14.
29. Borutaite V, Budriunaite A, Brown GC. Reversal of nitric oxide, peroxynitrite- and S-nitrosothiolinduced inhibition of mitochondrial respiration or complex I activity by light and thiols. Biochim Biophys 2000; Acta 1459:405–12.
30. Yu W, Naim JO, McGowan M, Ippolito K, Lanzafame RJ. Photomodulation of oxidative metabolism and electron chain enzymes in rat liver mitochondria. Photochem Photobiol 1997; 66:866–71.
31. Passarella S. He-Ne laser irradiation of isolated mitochondria. J Photochem Photobiol 1989; 3:642–43.
32. Lubart R, Eichler M, Lavi R, Friedman H, Shainberg A. Low-energy laser irradiation promotes cellular redox activity. Photomed Laser Surg 2005; 23:3–9.
33. Dawson JB, Barker DJ, Ellis DJ, Grassamt E, Cotterill JA, Fisher GW, et al. A theoretical and experimental study of light absorption and scattering by in vivo skin. Phys Med Biol 1980; 25:695–709.
34. Garcez AS, Ribeiro MS, Núñez SC. Laser de baixa potência: princípios básicos e aplicações clínicas na odontologia. Elsevier; 2012.

35. Simões A, Nicolau J, de Souza DN, Ferreira LS, Eduardo CP, Apel C, et al. Effect of defocused infrared diode laser on salivary flow rate and some salivary parameters of rats. Clin Oral Invest 2008; 12:25-30.
36. Loncar B, Stipetic MM, Baricevic M , Risovic D. The effect of low-level laser therapy on salivary glands in patients with xerostomia. Photomed Laser Surg 2011; 29:171–5.
37. Dirix P, Nuyts S, Van den Bogaert W. Radiation-induced xerostomia in patients with head and neck cancer: a literature review. Cancer 2006; 107:2525-34.
38. Eisbruch A, Kim HM, Terrell JE, Marsh LH, Dawson LA, Ship JA. Xerostomia and its predictors following parotid-sparing irradiation of head-and-neck cancer. Int J Radiat Oncol Biol Phys 2001; 50:695-704.
39. Henson BS, Eisbruch A, D'Hondt E, Ship JA. Two-year longitudinal study of parotid salivary flow rates in head and neck cancer patients receiving unilateral neck parotid-sparing radiotherapy treatment. Oral Oncol 1999; 35:234-41.
40. Stephens LC, King GK, Peters LJ, Ang KK, Schultheiss TE, Jardine JH. Acute and late radiation injury in rhesus monkey parotid glands. Evidence of interphase cell death. Am J Pathol 1986; 124:469-78.
41. Gavish L, Asher Y, Becker Y, Kleinman Y. Low level laser irradiation stimulates mitochondrial membrane potential and disperses subnuclear promyelocytic leukemia protein. Lasers Surg Med 2004; 35:369–76.
42. Lopes CO, Mas JR, Zângaro RA. Low level laser therapy in the prevention of radiotherapy induced xerostomia and oral mucositis. Radiol Bras 2006; 39:131–6.

43. Onizawa K, Muramatsu T, Matsuki M, Ohta K, Matsuzaka K, Oda Y, et al. Low-level (gallium-aluminum-arsenide) laser irradiation of Par-C10 cells and acinar cells of rat parotid gland. Lasers Med Sci 2009; 24:155-61.
44. Domínguez A, Ballesteros RE, Viáfara JH, Tamayo OM. Effect of low level laser therapy on dental pulp during orthodontic movement. World J Methodol 2013; 3:19-26.
45. Maegawa Y, Itoh T, Hosokawa T, Yaegashi K., Nishi M. Effects of near-infrared low-level laser irradiation on micro-circulation. Lasers Surg Med 2000; 27:427–37.
46. Henriksson R, Fröjd O, Gustafsson H, Johansson S, Yi-Qing C, Franzén L, et al. Increase in mast cells and hyaluronic acid correlates to radiation-induced damage and loss of serous acinar cells in salivary glands: the parotid and submandibular glands differ in radiation sensitivity. Br J Cancer 1994; 69:320-6.
47. Hakim SG, Kosmehl H, Lauer I, Nadrowitz R, Wedel T, Sieg P. A comparative study on the protection profile of lidocaine, amifostine, and pilocarpin on the parotid gland during radiotherapy. Cancer Res 2005; 65:10486-93.
48. Limesand KH, Avila JL, Victory K, Chang HH, Shin YJ, Grundmann O, et al. Insulin-like growth factor–1 preserves salivary gland function after fractionated radiation. Int J Radiat Oncol Biol Phys 2010; 78:579-86.
49. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132:27-42.
50. Li J, Shan Z, Ou G, Liu X, Zhang C, Baum BJ, et al. Structural and functional characteristics of irradiation damage to parotid glands in the miniature pig. Int J Radiat Oncol Biol Phys 2005; 62:1510-6.

51. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 2004; 5:897-907.
52. Desagher S, Martinou JC. Mitochondrial as the central control point of apoptosis. Trends Cell Biol 2000; 10:369-76.
53. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000; 6:513-6.
54. Konings AW, Coppes RP, Vissink A. On the mechanism of salivary gland radiosensitivity. Int J Radiat Oncol Biol Phys 2005; 62:1187-94.
55. Martin KL, Hill GA, Klein RR, Arnett DG, Burd R, Limesand KH. Prevention of radiation-induced salivary gland dysfunction utilizing a CDK inhibitor in a mouse model. PLoS One 2012; 7:e51363.
56. Xu J, Yan X, Gao R, Mao L, Cotrim A, Zheng C, et al. Effect of irradiation on microvascular endothelial cells of parotid glands in the miniature pig. Int J Radiat Oncol Biol Phys 2010; 78:897-903.
57. Limesand KH, Said S, Anderson SM. Suppression of radiation-induced salivary gland dysfunction by IGF-1. PLoS One 2009; 4:e4663.

ANEXO A

APROVAÇÃO DA COMISSÃO CIENTÍFICA E DE ÉTICA DA FACULDALDE DE ODONTOLOGIA DA PUCRS

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 23 de outubro de 2013

O Projeto de: Dissertação

Protocolado sob nº:	0050/13
Intitulado:	Efeito da radiação laser de baixa potência em glândulas parótidas de camundongos submetidos à radioterapia
Pesquisador Responsável:	Profa. Dra. Fernanda Gonçalves Salum
Pesquisadores Associados:	Monique Dossena Acauan
Nível:	Dissertação / Mestrado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *23 de outubro de 2013*

Este projeto deverá ser imediatamente encaminhado ao CEUA/PUCRS.

Profa. Dra. Luciane Macedo de Menezes
Coordenadora da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

CÓPIA

Av. Ipiranga, 6681, Prédio 06 sala 210
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900
Fone/Fax: (51) 3320-3538
e-mail: odontologia-pg@pucrs.br

ANEXO B

APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 106/13 - CEUA

Porto Alegre, 21 de novembro de 2013.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 13/00372, intitulado **"Efeito da radiação laser de baixa potência em glândulas parótidas de camundongos submetidos à radioterapia".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
Profa. Fernanda Gonçalves Salum
FO
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6681 - P. 99 - Portal Tecnopuc - sala 1512
CEP: 90619-900 - Porto Alegre/RS
Fone: (51) 3353-6365
E-mail: ceua@pucrs.br

ANEXO C

SUMISSÃO DO ARTIGO DE REVISÃO DA LITERATURA NO PERIÓDICO

ARCHIVES OF ORAL BIOLOGY

ees.aob.0.2eea5d.ad1b7225@eesmail.elsevier.com em nome de Archives of Oral Biology [AOB@e...

Para: Fernanda Goncalves Salum; fernanda_salum@hotmail.com

quarta-feira, 28 de janeiro de 2015 15

- Você encaminhou esta mensagem em 28/01/2015 16:19.

Archives of Oral Biology
Title: RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION: STRUCTURAL CHANGES, PATHOGENETIC MECHANISMS AND THERAPIES
Authors: Monique Dossena Acauan; Maria Antonia Z Figueiredo, Professor; Karen Cherubini, Professor; Ana Paula N Gomes, Ph.D; Fernanda G Salum, Ph.D
Article Type: Review Article

Dear Fernanda,

Your submission entitled "RADIOTHERAPY-INDUCED SALIVARY DYSFUNCTION: STRUCTURAL CHANGES, PATHOGENETIC MECHANISMS AND THERAPIES" has been received by Archives of Oral Biology.

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/aob/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal. Please do not hesitate to contact me if you have any queries.

Kind regards,

(On behalf of the Editors)

Archives of Oral Biology

ANEXO D

SUMISSÃO DO ARTIGO DE PESQUISA NO PERIÓDICO

JOURNAL OF BIOMEDICAL OPTICS

JBO MS #150117 Automated Notification of Manuscript Receipt

jbo@spie.org (jbo@spie.org) Adicionar aos contatos 27/02/2015
Para: fernanda_salum@hotmail.com

Dear Ms. Salum,

This is an automated email from the Journal of Biomedical Optics (JBO) to notify you that the following manuscript submission has been received by our system:

Manuscript Title: EFFECT OF LOW-LEVEL LASER THERAPY ON IRRADIATED PAROTID GLANDS - A STUDY IN MICE
Authors: Monique Acauan, Ana Paula Gomes, Aroldo Braga-Filho, Maria Antonia Figueiredo, Karen Cherubini, and Fernanda Salum
Paper Number: JBO 150117

Once the submission has been checked and validated by the journal staff, you will receive a formal acknowledgment officially confirming the submission. Please note that the staff operate on Pacific Standard Time and do not work on Saturdays or Sundays.

Sincerely,

JBO Editorial Office
SPIE
P.O. Box 10
Bellingham, WA 98227-0010

Phone: 360-676-3290